The Role of Host Factors in Semliki Forest Virus Infection

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Host factors play crucial roles in virus infections. Viruses exploit various cellular processes and are counteracted by an arsenal of host antiviral defenses. Characterization of these interactions is crucial for understanding the viral life cycle and developing novel antiviral treatments. Semliki Forest virus (SFV) is a positive-strand RNA alphavirus that has been used as a model virus for multiple clinically significant diseases such as lethal encephalitis. The aim of this thesis was to identify host factors that affect SFV infection to better understand the biology of SFV, and to provide candidate targets for therapies against more serious alphavirus infections.

Here I have conducted follow up studies on a previously performed genome-wide siRNA screen that hinted that a number of genes have novel functions in SFV infection. I used an automated high-throughput imaging-based approach to confirm the roles of these host factors in SFV infection. For comparison, I also used a similar strategy to test if these genes affect negative-strand RNA virus infections, using vesicular stomatitis virus (VSV). Additionally, I studied whether the host factors affecting SFV infections perform their roles in the entry and penetration, or post-penetration steps using a previously developed endocytic bypass assay.

I identified the γ-aminobutyric acid (GABA) transporter, SLC6A13, as a potential receptor for SFV. I also describe other novel genes that have roles in SFV or VSV infections. In addition, I show that TNP01, RPL18, ETF1, DMN2, and GNDPA1 promote, and HDAC6 counteracts SFV infection in the entry and membrane penetration steps. Furthermore, I report that in the later stages of the infection DDX54 boosts and EIF2B3, EIF4G1, PHB2, EDF1, DDX47, and DHX57 hinder SFV.

Avainsanat – Nyckelord – Keywords Host-virus interactions, siRNA, Automated high-throughput microscopy, RNA virus, SLC6A13, GAT-2, Semliki Forest virus, Vesicular stomatitis virus		
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Tiivistelmä – Referat – Abstract

Isäntäsolujen proteiinit ovat merkittävässä roolissa virusinfektioissa. Virukset hyödyntävät huomattavaa määrää isäntiensä luontaisista ominaisuuksista, ja solut puolustautuvat viruksia vastaan monin tavoin. Virusten ja isäntäsolujen välisten vuorovaikutusten selvittäminen on välttämätöntä virusten biologian ymmärtämiseksi. Näiden vuorovaikutusten tunteminen on myös tärkeää virusinfektioita hoidettaessa.

SFV (Semliki forest virus) on positiivisjuosteinen RNA-virus, joka toimii malliviruksena monille taudeille, kuten tappavalle virusaivokuumeelle. Tässä pro gradu -tutkielmassa tavoitteenani oli löytää uusia geenejä, jotka toimivat SFV-infektioissa. Akateemisen mielenkiinnon tyydyttämisen lisäksi uusien SFV-infektioihin liittyvien proteiinien tunnistaminen voi auttaa uusien hoitomuotojen kehittämiseksi vakavampia virustauteja vastaan.

Aikaisempi genominlaajuinen siRNA-kartoitus paljasti joukon geenejä, jotka saattavat vaikuttaa SFV-infektioihin. Tässä tutkielmassa selvitin näiden vaikutusta SFV-infektioihin käyttäen siRNA-teknologiaa sekä automatisoitua kuvantamista ja kuva-analyysiä. Vertailun vuoksi selvitin myös, kuinka nämä geenit vaikuttavat negatiivisjuosteisten RNA-virusten infektioihin käyttäen VSV-virusta (Vesicular stomatitis virus). Tämän lisäksi selvitin, toimivatko SFV-infektioon vaikuttavat proteiinit infektion alku- vai loppuvaiheissa.

Havaitsin, että γ-aminovoihappoa (GABA) kuljettava proteiini SLC6A13, saattaa toimia SFV-viruksen reseptorina. Löysin myös muita SFV- ja VSV-infektioihin vaikuttavia geenejä. Huomasin myös, että TNP01-, RPL18-, ETF1-, DMN2- ja GNDPA1-proteiineja tarvitaan SFV-infektion alkuvaiheissa. Tämän lisäksi selvitin, että DDX54-proteiini edistää ja EIF2B3-, EIF4G1-, PHB2-, EDF1-, DDX47 ja DHX57-proteiinit estävät SFV-infektiota vaikuttamalla sen myöhäisiin vaiheisiin.

Avainsanat – Nyckelord – Keywords
Virus-isäntäsoluvuorovaikutus, siRNA, Automatisoitu mikroskopia, RNA-virus, SLC6A13, GAT-2, Semliki forest virus, Vesicular stomatitis virus

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Declaration of authorship

The work presented in this thesis is my own and performed as a part of my master's thesis project, except for the following: I amplified and titered the Semliki Forest virus expressing ZsGreen, and validated the host factors involved in Semliki Forest virus infection as a part of the course "Research Project in Virology 1" (528041). Dr. Giuseppe Balistreri and Dr. Kirsi Hellström grew the BHK-21 cells, and Dr. Giuseppe Balistreri amplified the recombinant vesicular stomatitis virus expressing the green fluorescent protein.

Contents

1. List of abbreviations

AP2	Adaptor complex 2
AP2M1	Adaptor related protein complex 2μ 1
ATP	Adenosine triphosphate
ATP6V1B2	ATPase H+ transporting V1 subunit B2
ATP6V1G1	ATPase H+ transporting V1 subunit G1
BSA	Bovine serum albumin
CLTC	Clathrin heavy chain
CSPG5	Chondroitin sulfate proteoglycan 5
DDX	DEAD-box helicase
DHX	DEAH-box helicase
DMEM	Dulbecco's Modified Eagle's Medium
DMN2	Dynamin-2
EDF1	Endothelial Differentiation Related Factor 1
EIF2B3	Eukaryotic translation initiation factor 2B subunit γ
EIF4G1	Eukaryotic translation initiation factor γ 1
ESCRT	Endosomal sorting complexes required for transport
ETF1	Eukaryotic translation termination factor 1
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
GAT-2	GABA transporter 2
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GNPDA1	Glucosamine-6-phosphate deaminase 1
GTP	Guanosine triphosphate
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HDAC6	Histone deacetylase 6
HIV-1	Human immunodeficiency virus 1
IAV	Influenza A virus
kb	kilobase
KIF11	Kinesin family member 11
KPNB1	karyopherin subunit β 1
KREMEN2	Kringle containing transmembrane protein 2
MEM	Minimum Essential Medium
miRNA	Micro-RNA
mRNA	Messenger RNA
NRAMP2	Natural resistance associated macrophage protein 2
nsP	Non-structural protein
PBS	Phosphate-buffered saline
PFA	Para-formaldehyde
PHB2	Prohibitin-2
PM	Plasma membrane
PPBI	Cyclophilin B (human)
Ppbi	Cyclophilin B (mouse)
PVR	Polio virus receptor
+RNA	Positive-strand RNA
-RNA	Negative-strand RNA
RPL18	Ribosomal protein L18
RT	Room temperature
SFV	Semliki Forest virus
siRNA	Small interfering RNA
SLC6	Solute carrier family 6
SLC6A13	Solute carrier family 6 member 13
TNP01	Transportin-1
UPF1	Regulator of nonsense transcripts-1
UTR	Untranslated region
vATPase	Vesicular ATPase
VSV	Vesicular stomatitis virus

2. Introduction

2.1 Virus-host interactions

As obligate parasites, viruses require diverse cellular factors for the completion of their life cycles. Viruses use host factors to bind and enter cells as well as to replicate their genomes and produce new virions. On the other hand, a number of host proteins work to counteract viral infection.

To initiate successful infection, viruses need to bind to the surface of their host cells. In animal cells, this process is mediated by host's attachment factors and virus receptors, which are commonly cells surface glycoproteins and glycolipids (Smith & Helenius, 2004). Attachment factors bind the virus on the plasma membrane (PM) of the host, often via non-specific electrostatic interactions (Grove & Marsh, 2011) (Figure 1 A). This initial binding allows the bound virion to recruit the receptor molecules that initiate the entry process (Grove & Marsh, 2011) (Figure 1 B). Virus entry is usually mediated by endocytosis, a process by which the cell is able to internalize portions of the PM and extracellular solutes, such as nutrients and hormones (Marsh & Helenius, 2006). Multiple endocytosis mechanisms exist and they can be roughly divided to two categories based on the volume of the endocytic vesicle and the mechanism of vesicle formation (Doherty & McMahon, 2009). In "micropinocytosis", such as clathrin-mediated endocytosis, the vesicle is formed by invaginations of the PM and small volumes of the extracellular medium are internalized (Doherty & McMahon, 2009). Macropinocytic vesicles are the result of PM protrusions that "grab" extracellular medium or even other cells (phagocytosis) and then fuse back to the PM with their cargo (Doherty & McMahon, 2009). Different viruses, such as hepatitis C virus (HCV) (clathrin-mediated endocytosis) or Kaposi's sarcoma-associated herpesvirus (macropinocytosis) are able to exploit the full variety of these processes (Meertens *et al*, 2006; Raghu *et al*, 2009; Mercer *et al*, 2010).

Recruiting the endocytic machinery gives viruses multiple advantages over penetrating the PM directly. Internalizing the entire virion prevents the accumulation of potentially antigenic viral components on the host surface, gives

the virus a convenient way to infiltrate deep into the cell, and gives the virus access to intracellular compartments where chemical cues destabilize the viral particle and initiate the disassembly process known as uncoating (Marsh & Helenius, 2006). These cues are either changes in the pH of the endosome or the action of endosomal enzymes (Grove & Marsh, 2011). The conditions of the endosome cause conformational changes in the viral fusion proteins, which allows the virus to penetrate the endosomal membrane into the cytoplasm (Grove & Marsh, 2011). This results in the delivery of the viral nucleocapsid into the site of uncoating and/or replication (Grove & Marsh, 2011) (Figure 1 D).

The next step in the viral life cycle is the transcription and translation of viral messenger RNAs (mRNAs), or in the case of positive-strand (+RNA) viruses, the direct translation of the viral genome. RNA viruses use their own RNA-dependent RNA polymerases for transcription but they use host machinery for translation, which leads to a translational competition between host and viral RNAs (Walsh & Mohr, 2011) (Figure 1 E). This interaction is further complicated by host antiviral defenses that aim to shut down translation (Walsh & Mohr, 2011). Therefore it is understandable that viruses have evolved myriad mechanisms to usurp the protein synthesis machinery of the host and to keep it operational despite host antiviral response (Walsh & Mohr, 2011).

Once the cell is under viral control, the final stage of the infection begins. The viral genome is replicated and progeny virions are assembled by exploiting host resources and structures, such as the PM, the endoplasmic reticulum, or the Golgi apparatus (Kuismanen *et al*, 1982; Gosert *et al*, 2003; Spuul *et al*, 2010) (Figure 1 E). Once the new generation of virions is ready, they exit the cell using various strategies. Viruses may, for example, use the host's ESCRT (endosomal sorting complexes required for transport) system and bud from the PM (Votteler & Sundquist, 2014), use an exocytosis-like pathway (Johnson & Baines, 2011), or cause the lysis of the host cell (Tollefson *et al*, 1996) (Figure 1 F).

As virus infection is highly dependent on host functions, elucidating the interplay between viral and cellular factors is crucial in understanding the biology of

viruses. Observing host-virus interactions also plays a key role in the development of new treatments and therapies. In addition, by following the different stages of virus infections, it is possible to assign novel functions to poorly characterized cellular factors.

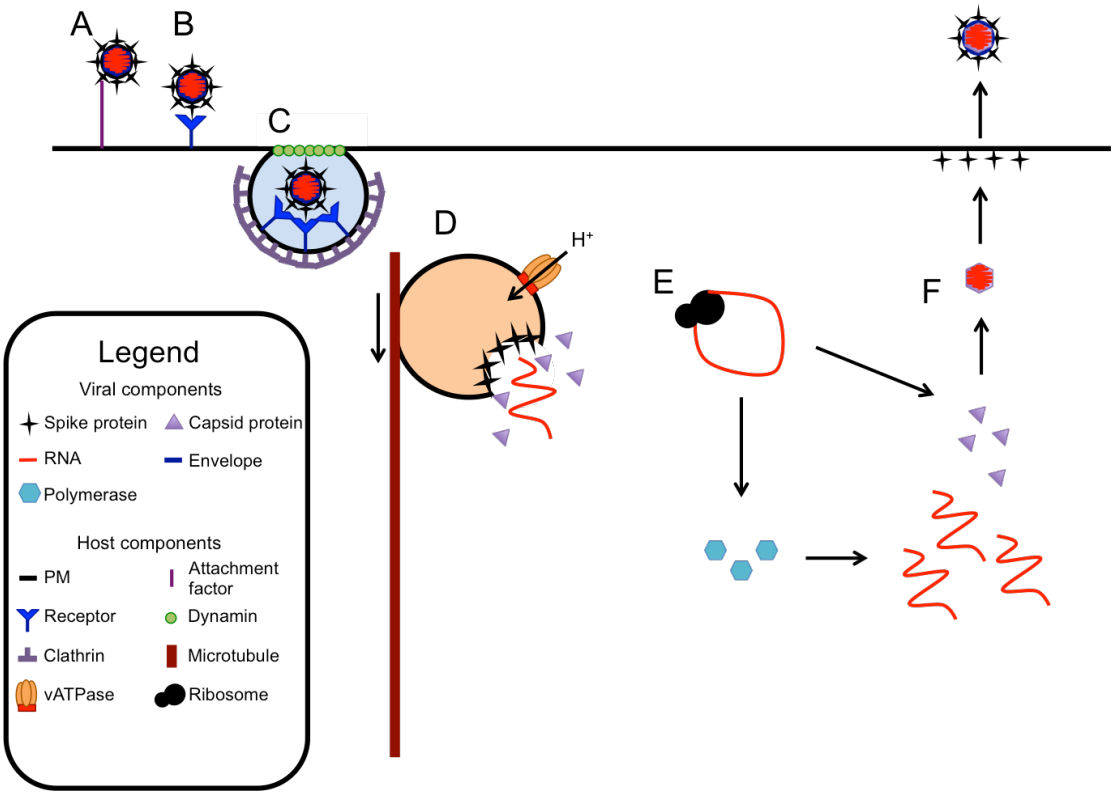

Figure 1. The life cycle of an endocytosis-utilizing enveloped virus. A: The virus infection begins with non-specific binding on host attachment factors such as heparan sulphate. **B:** Non-specific binding leads to receptor recruitment **C:** Receptor binding is often multivalent and triggers the endocytosis event. **D**: The endosome carries the virus deeper into the cell using the tubular network. Endosomal enzymes and/or the acidification of the endosome by the action of host vesicular ATPases (vATPases) leads to a conformational change in the viral spike proteins. This allows a membrane fusion event to occur, which releases the nucleocapsid into the cytosol. The viral genome is uncoated and transported to its replication site. **E**: Viral RNA outcompetes host mRNA by limiting host transcription or preventing host translation. This leads to the production of viral proteins and the replication of the viral genome. **F:** The viral components assemble into nucleocapsids, which exit from the cells e.g. by budding from the PM.

2.2 Semliki Forest virus and vesicular stomatitis virus

Semliki Forest virus (SFV) is one of the best-studied members of the genus *Alphavirus*. Like other alphaviruses, it is a +RNA virus that infects both invertebrate and vertebrate hosts (Griffin, 2013). SFV virions are enveloped and have a diameter of 70 nm, with icosahedrally symmetric nucleocapsids (Mancini *et al*, 2000). SFV enters host cells using endocytosis and penetrates the endocytic vesicle after a low pH-induced conformational change in the SFV spike glycoproteins leads to the fusion of viral and endosomal membranes (Helenius *et al*, 1980; White *et al*, 1980; Fuller *et al*, 1995). Once in the cytosol, the nucleocapsid is immediately engaged by host ribosomes, which triggers the

uncoating of the nucleocapsid and leads to viral translation (Singh & Helenius, 1992). The low pH of the endosomes (<6.5) is crucial for SFV penetration, and treatments with small molecule inhibitors that neutralize endosomal acidification block virus penetration and infection (Helenius *et al*, 1980). The role of endosomal acidification in SFV infection has been confirmed by small interfering RNA (siRNA)-mediated depletions of different subunits of the host vacuolar ATPase (vATPase), a membrane pump responsible for the acidification of the endosomes (Balistreri *et al*, 2014).

The SFV genome is approximately 11.5 kilobases (kb) long and contains two open reading frames and 3' and 5' untranslated regions (UTR) (European Nucleotide Archive, http://www.ebi.ac.uk/ena/data/view/X04129, Leinonen *et al*, 2011). The early genes of the SFV genome are translated directly from the genome, which is later replicated via a -RNA intermediate that is also used to produce subgenomic RNA, which is translated to the structural genes (Kuhn, 2013). Like with other alphaviruses, SFV infection results in a global inhibition of host transcription and translation (Fros & Pijlman, 2016). The viral non-structural protein (nsP) 2 seems to play an important role in this process (Fros & Pijlman, 2016). To counteract the host's antiviral defense, the SFV sub-genomic RNA contains a 5' hairpin loop that allows translation of SFV capsid and envelope proteins even when cap-dependent translation is inhibited due to the host-induced phosphorylation of the translation factor eIF2α (Fros & Pijlman, 2016).

Vesicular stomatitis virus (VSV) is a negative-strand (-RNA) virus spread by insect vectors and it belongs to the family *Rhabdoviridae* (Mead *et al*, 2000; Lyles *et al*, 2013). It usually infects livestock but can occasionally cause disease in humans (Lyles *et al*, 2013). The genome of VSV is about 11.2 kb in length, and it lacks 5' and 3' UTRs (Lyles *et al*, 2013; National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/nuccore/J02428, Coordinators, 2017). VSV virions are approximately 200 nm long with bullet-shaped morphology and the nucleocapsids follow helical symmetry (Ge *et al*, 2010). Like SFV, VSV enters host cells via endocytosis and the fusion of the host and VSV membranes dependent on the low pH of the endosome (Regan & Whittaker,

2013). However, VSV fusion occurs later than SFV fusion and it is mediated by a two-step fusion program (Le Blanc *et al*, 2005). First the VSV nucleocapsid is released to the lumen of an intra-endosomal vesicle and it enters the cytoplasm later, following a back-fusion of the intra-endosomal vesicle (Le Blanc *et al*, 2005). The transcription and replication of VSV follows the general scheme of non-segmented -RNA viruses (Lyles *et al*, 2013). The genome is used as a template for viral mRNAs and a +RNA-antigenome, which is used as template for the new -RNA genomes.

2.3 The role of host factors in SFV infection

Previously, a genome-wide siRNA screen was performed to identify host factors that affect SFV infection (Balistreri *et al*, 2014). The screen implicated a number of genes ("hits") (Table 1). Following the screen, the roles of UPF1, ATP6V1B2, and ATP6V1G1 in SFV infection were characterized (Balistreri *et al*, 2014).

Table 1. Genes implicated in SFV infection by the genome-wide siRNA screen.

Gene symbol	Function	Gene ID*	Gene symbol	Function	Gene ID*
PHB2	Intracellular signaling	11331	DDX31	DEAD-box helicase	64794
EDF1	Transcriptional coactivation	8721	DDX41	DEAD-box helicase	51428
SLC6A13	GABA & taurine transport	6540	DDX43	DEAD-box helicase	55510
EIF2B3	Translation initiation	8891	DDX47	DEAD-box helicase	51202
ETF1	Translation termination	2107	DDX54	DEAD-box helicase	79039
EIF4G1	Translation initiation	1981	DHX37	DEAH-box helicase	57647
DDX18	DEAD-box helicase	8886	DHX57	DEAH-box helicase	90957
PVR	Poliovirus receptor	5817	KREMEN2	Transmembrane receptor	79412
CSPG5	Chondroitin sulfate proteoglycan 5	10675	HDAC6	Deacetylation of various proteins	10013
GNPDA1	Glucosamine-6-phosphate deaminase	10007	DNM2	Endocytosis	1785
RPL18	Ribosomal protein	6141	AP2M1	Vacuolar ATPase activation	1173
UPF1	mRNA quality control, antiviral activity	5976	CLTC	Endocytosis	1213
ATP6V1B2	Vesicle acidification	526	KPNB1	Nuclear localization	3837
ATP6V1G1	Vesicle acidification	9550	TNPO1	Intracellular localization	3842

* O'Leary *et al*, 2016

The first aim of this thesis was to confirm the roles of the hits in SFV infection and investigate if their effect was specific to SFV. This was done using siRNA-mediated knockdown and high-throughput imaging to determine if the hits affected SFV and VSV infections. The second aim was to assign a role in the early (entry and penetration), or later (post-penetration) stages of SFV infection for the confirmed host factors. This was performed using a similar strategy as above combined with an assay to bypass the endocytosis step of SFV infection (White *et al*, 1980) (Figure 2).

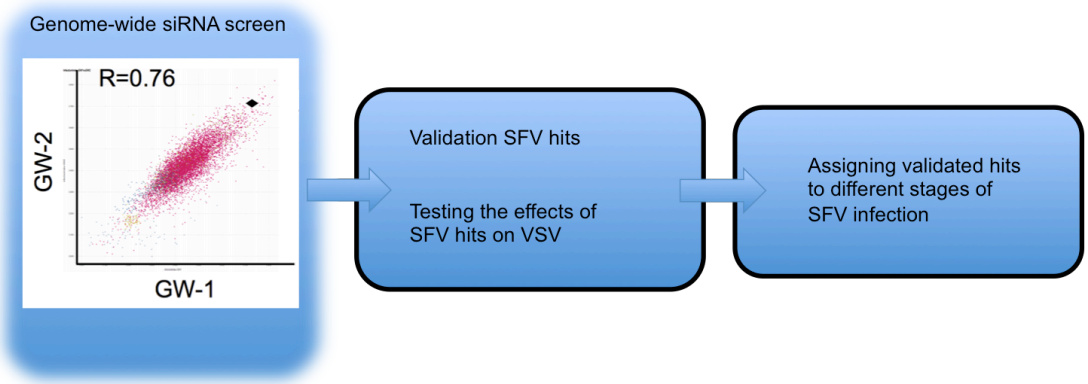

Figure 2. The aim of this thesis. Previously, a genome-wide image-based siRNA screen identified numerous candidates (or "hits") for SFV infection affecting host genes. In this thesis, I have analyzed their role in virus infections using small-scale image-based siRNA knockdown studies. I confirmed if these hits had a role in SFV infection and tested if they affected VSV infection for comparison. For the genes affecting SFV infection, I further studied if their role was related to either the entry and penetration, or post-penetration stages of the infection. The genome-wide screen image was kindly provided by Giuseppe Balistreri.

In this thesis I describe novel genes affecting SFV infection, as well as novel host factors with a role in VSV infection. I also pinpoint the effects of genes involved in SFV infection to entry and penetration, or post-penetration steps. In addition, I show that SLC6A13, a membrane-spanning γ-aminobutyric acid (GABA) and taurine transporter (Kristensen *et al*, 2011; Zhou *et al*, 2012), is needed for the early events of SFV infection, but has no role in VSV infection, and is therefore a candidate receptor for SFV.

3. Materials and methods

3.1 Cell lines and viruses

HeLa cells (ATCC) were cultured using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, D7777) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, F9665), GlutaMAX (Gibco, 35050-061), Non-essential Amino Acids Solution (Sigma-Aldrich, M7145), and Antibiotics & Antimycotics Solution (Sigma-Aldrich, A5955) (HeLa growth medium). BHK-21 cells (ATCC) were culture using Minimum Essential Medium (MEM) (Gibco, 61100-087) supplemented with 10 % FBS and GlutaMAX. The cells were grown at +37 °C with a 5% CO_2 atmosphere.

SFV expressing ZsGreen protein fused with the virus nsP 3 (SFV-ZsGreen) (Spuul *et al*, 2010) and rVSV-GFP, a VSV strain expressing green fluorescent protein (GFP) (Pelkmans *et al*, 2005) have been previously described. The viruses were originally produced at ETH Zurich (Switzerland) and were provided by Dr. Balistreri with permission from professor Ari Helenius.

3.2 Virus production and titration

Virus inocula were prepared in MEM supplemented with 20 mM HEPES (pH 7.2), GlutaMAX, and 0.2 % bovine serum albumin (BSA) and confluent BHK-21 cells were washed twice with phosphate-buffered saline (PBS) and infected with either SFV-ZsGreen or rVSV-GFP using an MOI of 0.01 by replacing the old media with the virus inocula. The infected cells were incubated at +37 °C with a 5% CO_2 atmosphere. The media were collected after 22h and centrifuged at 3900 rpm for 20 min (SFV-ZsGreen) or 10 min (rVSV-GFP) at +4 °C (Eppendorf Centrifuge 5810R) to eliminate cell debris. The supernatants were collected, aliquoted and stored at -80 °C.

HeLa cells were seeded onto black clear-bottom 96-well plates (Corning, 07-200-568) at a density of 10 000 cells / well and grown for 16–20 h at +37 °C with a 5% CO_2 atmosphere. The cells were washed with DMEM supplemented with GlutaMAX and Antibiotics & Antimycotics Solution (SFV-ZsGreen) or RPMI-1640

medium (ICN, 1060122) containing 20 mM HEPES pH 7.0 and GlutaMAX (rVSV-GFP) (100 µl / well). The cells where then infected with 1:2 (SFV-ZsGreen) or 1:10 (rVSV-GFP) serial dilutions in duplicate using 100 µl of virus inocula in corresponding media The infected cells were grown for 6 h (SFV-ZsGreen) or 7 h (rVSV-GFP) at +37 °C with a 5% CO_2 atmosphere.

After the incubation, the media were aspirated and the cells were fixed for 20 min at room temperature (RT) using 4 % para-formaldehyde (PFA) in PBS (100 µl/ well). The fixed cells were washed 3 times with PBS (100 µl/ well). The cells were permeabilized and the nuclei stained by incubating the cells for 10 min at RT in 100 µl of PBS containing 0.2 % Triton X-100 and Hoechst DNA stain (1 µg/ ml) per well. The cells were then washed 3 times with PBS (100 µl/ well) and the plates covered with Black TopSeal-A plate seals (PerkinElmer, 6050173) and stored at +4 °C. The plates were imaged with a high-content Cellinsight Imager microscope (Thermo Fisher) at the Light Microscopy Unit, Institute of Biotechnology. 16 images were taken per well, using both the 386 nm and the 485 nm filters to visualize the fluorescence signal of the Hoechst and the ZsGreen or GFP. The images were analyzed using the open source Cellprofiler 2 software (Carpenter *et al*, 2006, www.cellprofiler.com) (see below).

3.3 Virus infections of siRNA-transfected cells

HeLa cells were reverse transfected using pooled siRNAs (Dharmacon SMARTpool siRNAs) on a black clear-bottom 96-well plate using a separate well for each siRNA pool. The siRNA pools contained a mixture of 4 different oligonucleotides against non-overlapping regions of each target gene in equimolar concentrations. The siRNA pools targeted the genes of interest as well as control genes (KIF11, Ppbi and PPBI) (table S1). The siRNA pool against KIF11 was used as a transfection control. Since the product of this gene is essential for cell survival, monitoring cell death was used as a means to make sure the transfection was efficient (>98% cell death indicated successful transfection). A mix of 4 different non-specific (or “scrambled”) siRNAs was used as a negative control in four separate wells. The siRNAs against Ppbi and PPBI were supplied by the manufacturer as easily quantifiable transfection controls.

For each well, 10 µl of a siRNA stock (100 nM) was mixed with 10 µl of DMEM containing 0.1 µl of Lipofectamine 2000 (Invitrogen, 11668-019) and the plate was incubated at RT for 30–60 min. Then for each well, 2000 HeLa cells suspended in 80 µl of DMEM were mixed with the siRNA–Lipofectamine 2000 mix yielding a final siRNA concentration of 10 nM per well. The plate was then incubated at +37 °C with a 5% CO_2 atmosphere for 6 h, after which, the medium was replaced with HeLa growth medium (200 µl/ well) and the cells were grown at +37 °C with a 5% CO_2 atmosphere.

After 72 h the old media were removed and the cells were washed with either DMEM supplemented with GlutaMAX and Antibiotics & Antimycotics Solution (SFV-ZsGreen) or RPMI-1640 medium (ICN, 1060122) containing 20 mM HEPES pH 7.0 and GlutaMAX (rVSV-GFP) (100 µl / well). The cells were then infected with 100 µl / well of virus inocula containing $6*10^4$ pfu of either SFV-ZsGreen or rVSV-GFP in corresponding media. The cells were incubated at +37 °C with a 5% CO_2 atmosphere for 5 h (SFV-ZsGreen) or 6.5 h (rVSV-GFP) and the plates were fixed, stained, imaged, and analyzed as above. Three biological repetitions per virus were performed.

3.4 Endocytic bypass assay

HeLa cells were reverse transfected as above. 72 h after transfection, the plate was placed on ice and cells washed with ice-cold RPMI-1640 medium containing 20 mM HEPES, pH 7.0 (100 µl / well) and infected on ice with $6*10^4$ pfu of SFV-ZsGreen diluted in the same medium (ice-cold) (100 µl / well). The plate was incubated on ice for 1 h and the medium was removed from the wells. To allow virus fusion with the PM, the cells were treated with RPMI-1640 medium containing 10 mM MES buffer, pH 5.5 for 90 s at +37 °C. The medium was aspirated and replaced with HeLa growth medium supplemented with 20mM NH_4Cl and 20mM HEPES, pH 7.2 (200 µl / well). The plate was incubated at +37 °C with 5% CO_2 atmosphere for 4 h and the plate was fixed, stained, imaged, and analyzed as above. Controls included wells that were not infected, wells treated with pH 7.0 medium instead of the pH 5.5 medium, and wells treated with pH 7.0

medium and HeLa growth medium instead of the pH 5.5 medium and HeLa growth medium with NH_4Cl, respectively. Three biological repetitions were performed.

3.5 Automated high-throughput image analysis

The Cellprofiler 2 software (Carpenter *et al*, 2006, www.cellprofiler.com) was used to determine the percentage of infected cells in each well. In each image, the number of cells was determined by detecting the 386 nm fluorescence signal of the stained nuclei, designated "primary objects" (Figure 3 A & B). SFV-ZsGreen produces ZsGreen-labeled nsP3, thus ZsGreen-expressing cells are infected with SFV. For the SFV experiments the perimeter of each detected nucleus was digitally expanded to include a portion of the cytoplasm around the nucleus, designated "secondary objects" (Figure 3 C, D, E & F). This was done to detect the ZsGreen signal from the cytoplasm, where the SFV protein synthesis occurs. The mean fluorescence signal on the 485 nm channel (ZsGreen) was measured for each secondary object. For the rVSV-GFP-infected cells the mean fluorescence signal on the 485 nm channel (GFP) was measured within each primary object (nucleus), as the GFP protein encoded by the virus was synthesized in the cytoplasm and freely diffused into the nucleus of the host indicating successful viral translation.

Using a threshold value of mean 485 nm fluorescence, the cells were classified in two categories: infected (above threshold) and non-infected (below threshold) (Figure 4 G). Non-infected cells were always included to calibrate the threshold settings for each imaged plate. Once all images in each well of a 96 well plate were analyzed, the final results were expressed as total infected cells per well. To calculate virus titers, the estimated amount of cells per well (16,000) (Rafferty, 1985) was multiplied with the percentage of infected cells to determine the amount of infectious virions per well. In the siRNA experiments, cells transfected with non-targeting (or "scrambled") siRNAs were used as controls. The mean number of infected cells in the scrambled controls was set as 1 and the infection percentages in the remaining transfected wells were normalized accordingly. The mean infection percentage per gene was calculated from three biological

repetitions. A difference of 2 or more standard deviations from controls was considered to be significant.

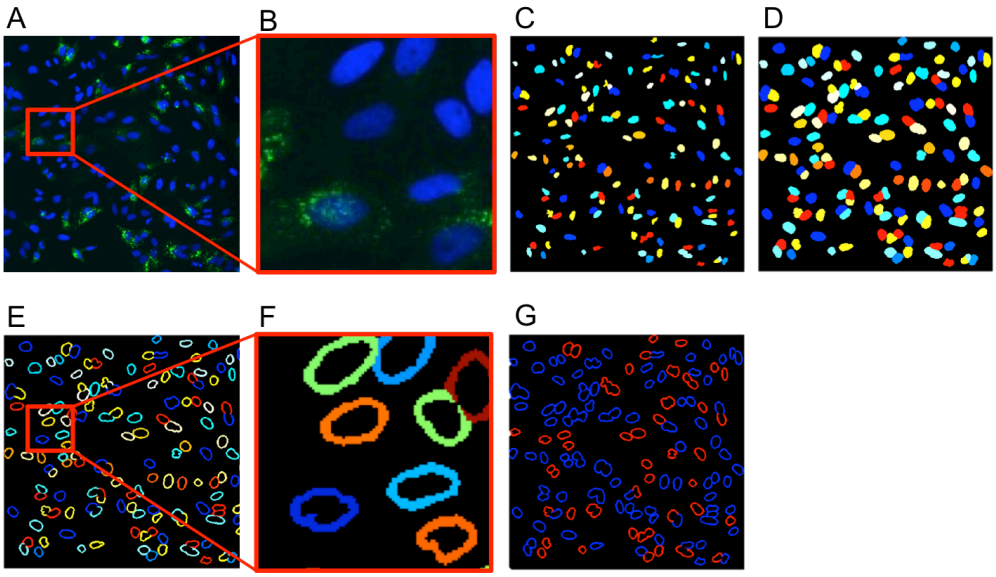

Figure 3. Automated detection of infected cells using Cellprofiler 2. A & B: Fluorescence signal detected using 395 nm (blue) and 485 nm (green) filters from SFV-infected cells pre-treated with scrambled control siRNA. **C:** Automatically detected nuclei (pseudocoloured 'primary objects'). **E–F:** Pseudocoloured secondary objects obtained by digitally expanding the primary objects (D) and subtracting the area of the primary objects. **G:** The secondary objects were used to automatically classify infected (red) or non-infected (blue) cells, using the mean fluorescence intensity from the 485 nm channel (G).

4. Results

The aim of this thesis was to study the effects of knocking down host factors previously implicated in SFV infection, and compare those effects to VSV infection. The genes affecting SFV infection were further characterized by studying if they affect entry and penetration, or post-penetration stages of SFV infection.

4.1 Knocking down previously implicated host factors affects SFV and VSV infections

To determine which of the previously implicated genes affect SFV and VSV infections, I used siRNA-mediated knockdown, high-throughput imaging and automated image analysis. I found that both SFV and VSV infection could be reduced significantly by knocking down ATP6V1B2 and ATP6VG1, two subunits of a vacuolar ATPase know to affect SFV infection (Balistreri *et al*, 2014) as well

as ribosomal protein L18 (RPL18), a part of the 60 S ribosomal subunit (de la Cruz *et al*, 2015) (Figures 4 & 5). Both SFV and VSV infections were affected by the knockdown of transportin-1 (TNP01), an intracellular transport molecule (Twyffels *et al*, 2014), prohibitin 2 (PHB2), an intracellular signaling protein (Bavelloni *et al*, 2015), and DEAH-box helicase (DHX) 37, a poorly characterized RNA-helicase (Gene ID: 57647) (Figures 4 &5). However, the knockdown of these genes had the opposite effects on SFV and VSV infections, as TNP01 depletion decreased SFV infection and increased VSV infection significantly, and in the case of PBH2 and DHX37 depletions SFV infection increased and VSV infection decreased significantly (Figures 4 & 5).

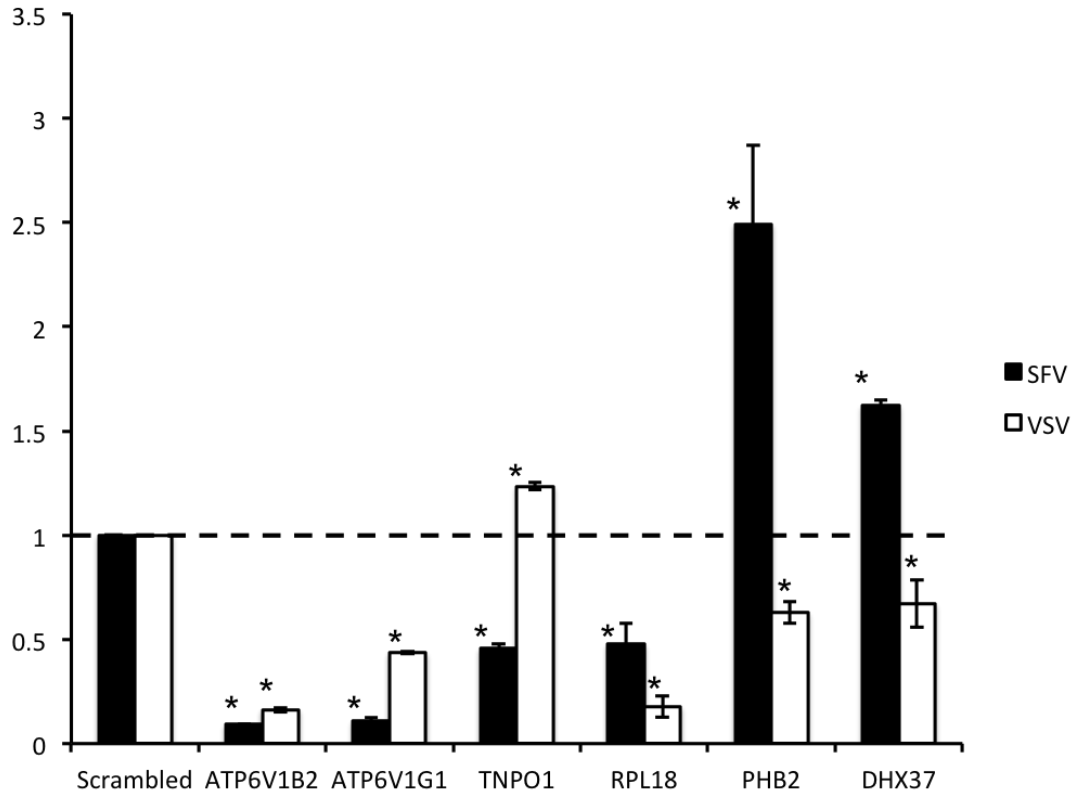

Figure 4. Relative infection percentages in cells depleted of host factors that affect both SFV and VSV infections. The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen (black) or VSV-GFP (white) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control.

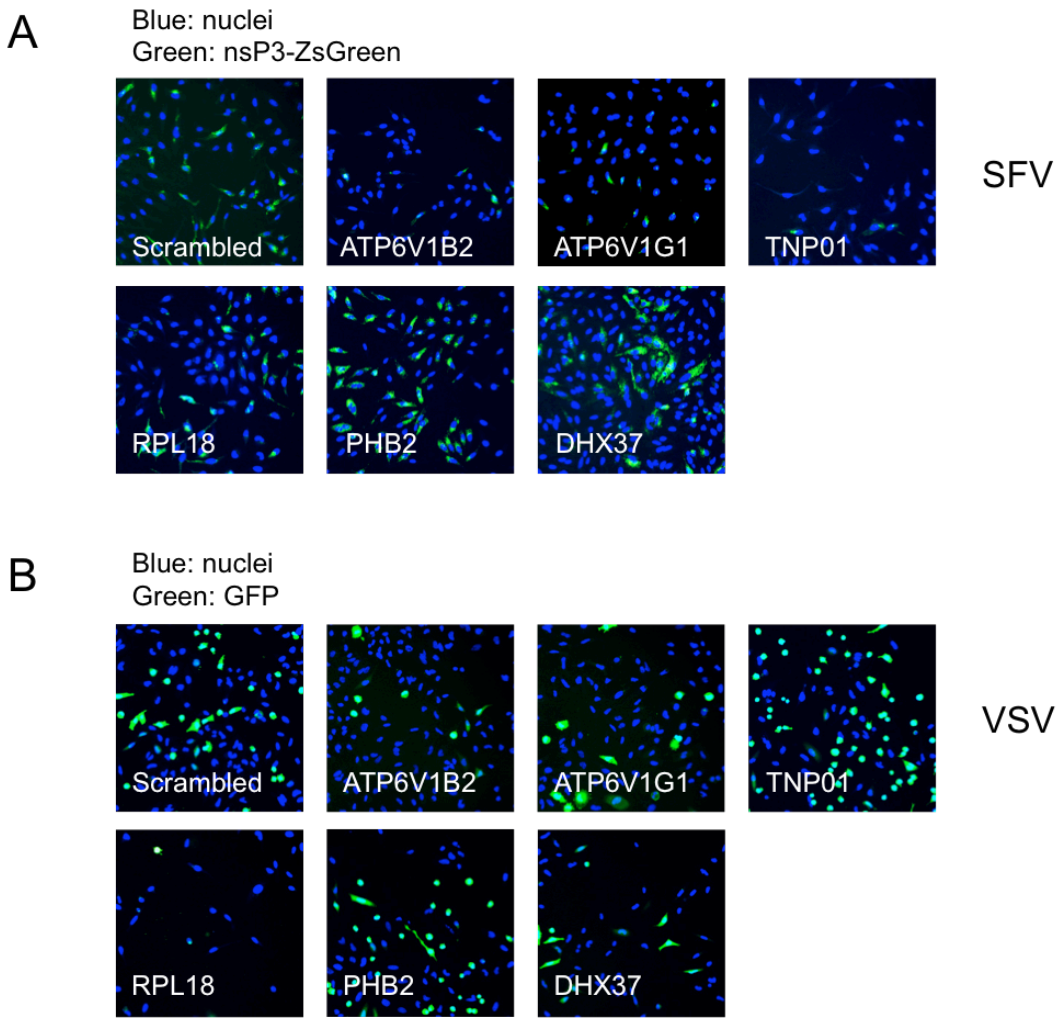

Figure 5. Representative images of cells depleted of host factors that affect both SFV and VSV infections. A: HeLa cells infected with SFV-ZsGreen. Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images. **B:** HeLa cells infected with VSV-GFP. Nuclei are shown in blue and GFP, indicating VSV infection, is shown in green. The siRNAs used are indicated by the text in the images.

By depleting cells of solute carrier family 6 member 13 (SLC6A13), a GABA and taurine transporter (Kristensen *et al*, 2011; Zhou *et al*, 2012), GNPDA1, a poorly characterized glucosamine-6-phosphate deaminase (Wolosker *et al*, 1998), DEAD-box helicase (DDX) 54, an RNA-helicase that has a transcription-regulating role in cells (Rajendran *et al*, 2003; Kanno *et al*, 2012), and dynamin-2 (DMN2), a protein involved in endocytosis (Kasai *et al*, 1999), SFV infection could be significantly reduced (Figures 6 & 7). On the other hand, the knockdown of eukaryotic translation initiation factor 2B subunit γ (EIF2B3) and eukaryotic translation initiation factor γ 1 (EIF4G1) (Walsh & Mohr, 2011), eukaryotic translation termination factor 1 (ETF1) (Taylor *et al*, 2012), UPF1, an RNA-helicase that prevents SFV infection (Balistreri *et al*, 2014), DDX47 and DHX57,

predicted RNA helicases (Gene IDs: 51202 and 90957, respectively), histone deacetylase 6 (HDAC6), a multifunctional cellular deacetylase (Hubbert *et al*, 2002), and endothelial differentiation-related factor 1 (EDF1), a transcriptional co-activator (Kabe *et al*, 1999) increased SFV infectivity significantly (Figures 6 & 7). The depletion of these SFV-affecting genes had no significant effect on VSV infection (Figures 6 & 7).

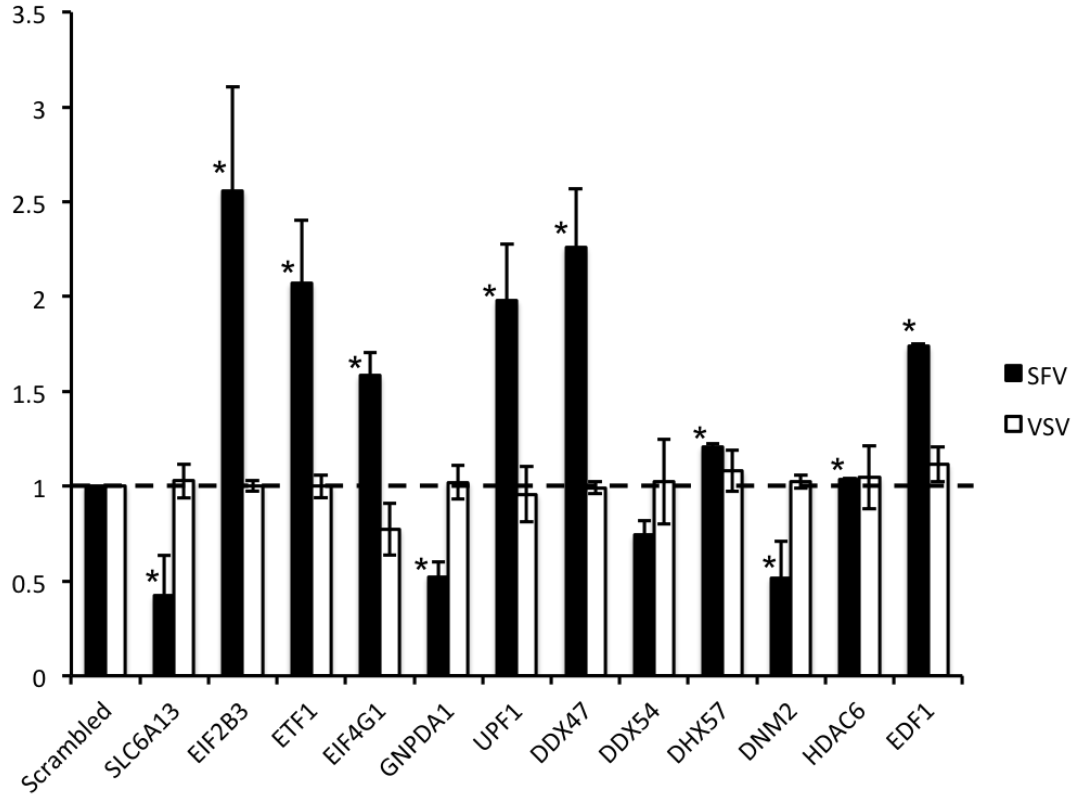

Figure 6. Relative infection percentages in cells depleted of host factors that affect only SFV infection. The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen (black) or VSV-GFP (white) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control.

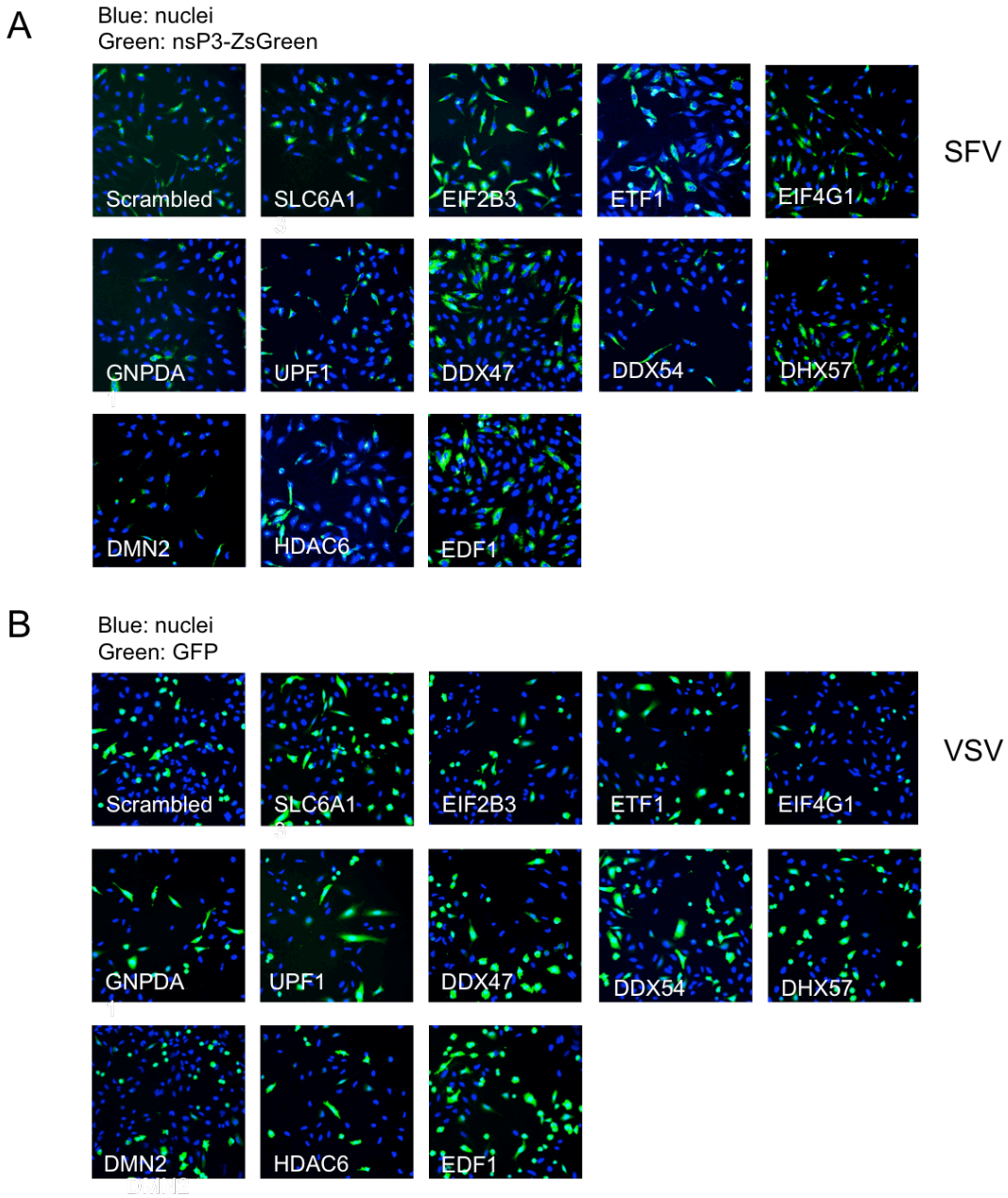

Figure 7. Representative images of cells depleted of host factors that affect only SFV infection. A: HeLa cells infected with SFV-ZsGreen. Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images. **B:** HeLa cells infected with VSV-GFP. Nuclei are shown in blue and GFP, indicating VSV infection, is shown in green. The siRNAs used are indicated by the text in the images.

VSV infectivity was significantly increased by the depletion of the poliovirus receptor (PVR) (Mendelsohn *et al*, 1989), a Wnt/β-catenin-signaling receptor, kringle containing transmembrane protein 2 (KREMEN2) (Mao *et al*, 2002), and adaptor related protein complex 2μ 1 (AP2M1), an adaptor of endocytic vesicles and vATPases, (Heinaman, 1995) (Figures 8 & 9). Knocking down DDX18, a poorly understood RNA-helicase (Dubaele & Chène, 2007) and karyopherin

subunit β 1 (KPNB1), a subunit of a nuclear import protein (Görlich *et al*, 1995) decreased VSV infectivity significantly (Figures 8 & 9). Curiously, siRNAs against both mouse and human cyclophilin B (Ppib and PPBI, respectively) supplied as transfection controls by the manufacturer increased VSV infectivity significantly (Figures 8 & 9). Cyclophilin B is a multifunctional signaling and anti-inflammatory protein (Hoffmann & Schiene-Fischer, 2014). None of these genes had a significant effect on SFV infectivity (Figures 8 & 9).

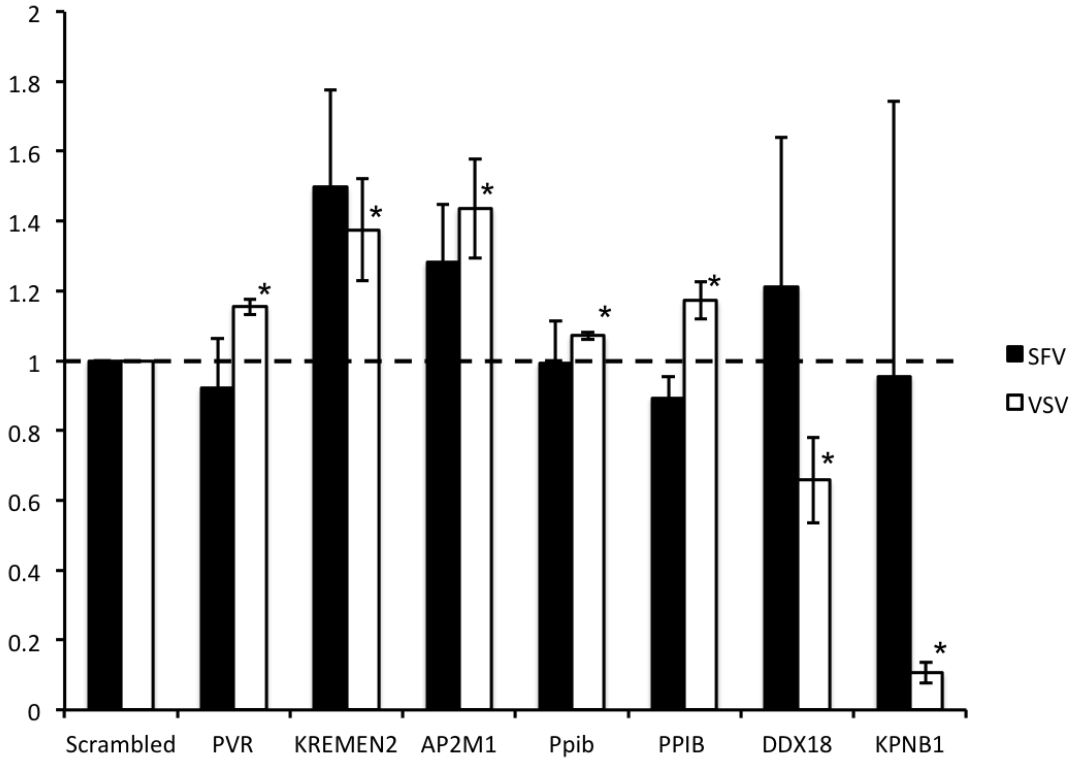

Figure 8. Relative infection percentages in cells depleted of host factors that affect only VSV infection. The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen (black) or VSV-GFP (white) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control.

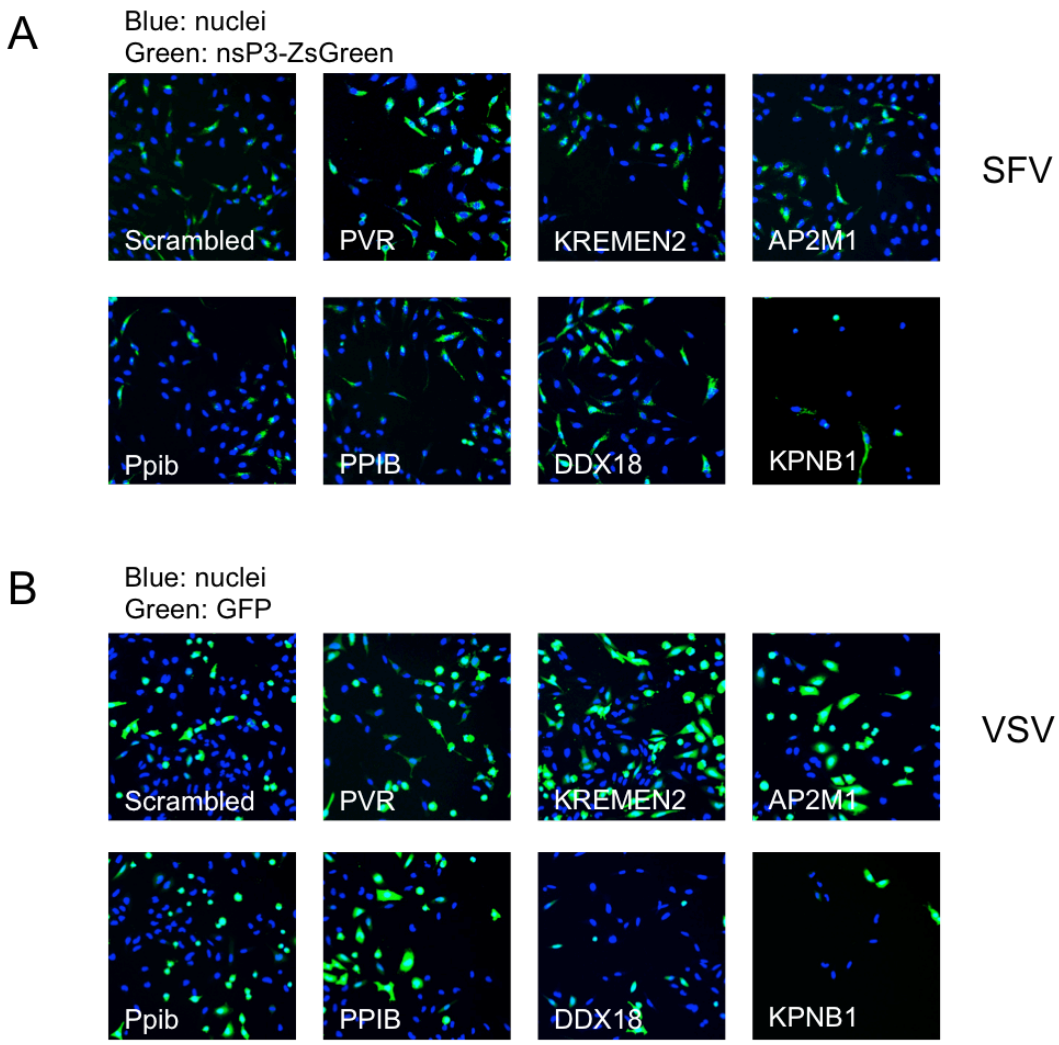

Figure 9. Representative images of cells depleted of host factors that affect only VSV infection. A: HeLa cells infected with SFV-ZsGreen. Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images. **B:** HeLa cells infected with VSV-GFP. Nuclei are shown in blue and GFP, indicating VSV infection, is shown in green. The siRNAs used are indicated by the text in the images.

The depletion of some of the genes implicated by the genome-wide siRNA screen did not have a significant effect on SFV or VSV infection (Figures 10 & 11). These included chondroitin sulfate proteoglycan 5 (CSPG5), a cell surface protein expressed in the brain (Watanabe *et al*, 1995), DDX31, an RNA-helicase implicated in renal cancer (Fukawa *et al*, 2012), DDX41, an RNA helicase with a role in cellular immunity (Jiang *et al*, 2017), DDX43, a RNA and DNA helicase (Tanu *et al*, 2017), and clathrin heavy chain (CLTC), a well-studied endocytosis molecule (Doherty & McMahon, 2009).

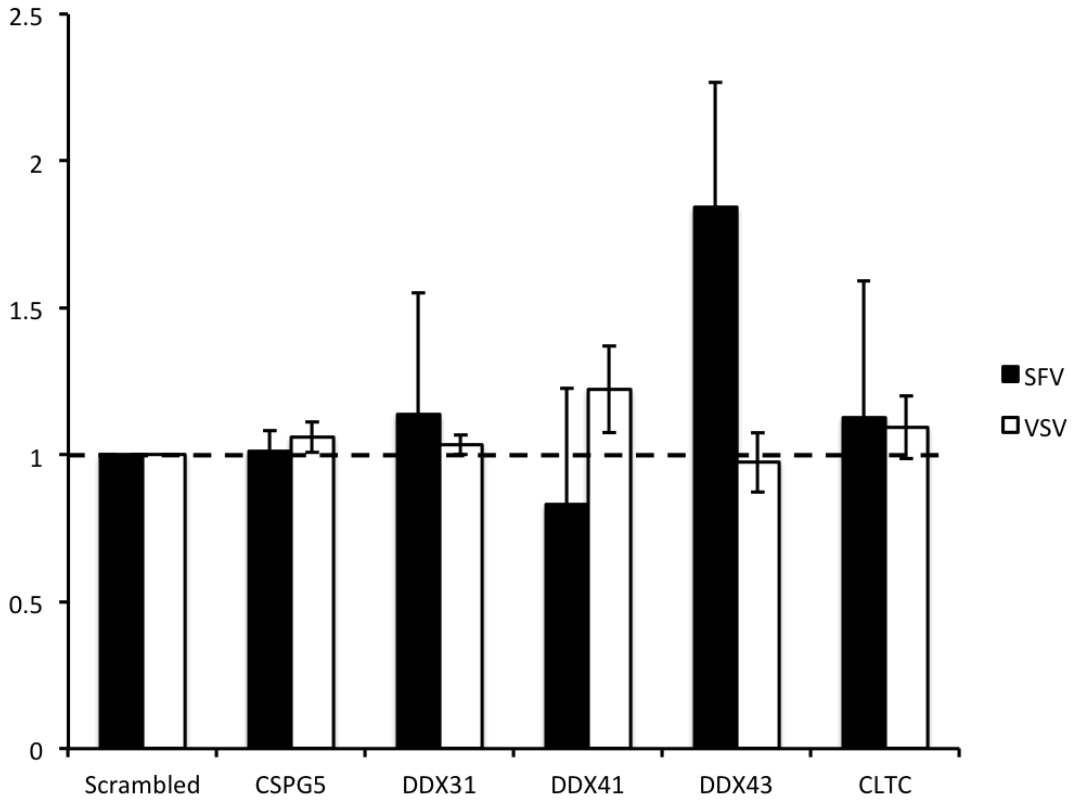

Figure 10. Relative infection percentages in cells depleted of host factors that do not affect SFV of VSV infections. The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen (black) or VSV-GFP (white) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control.

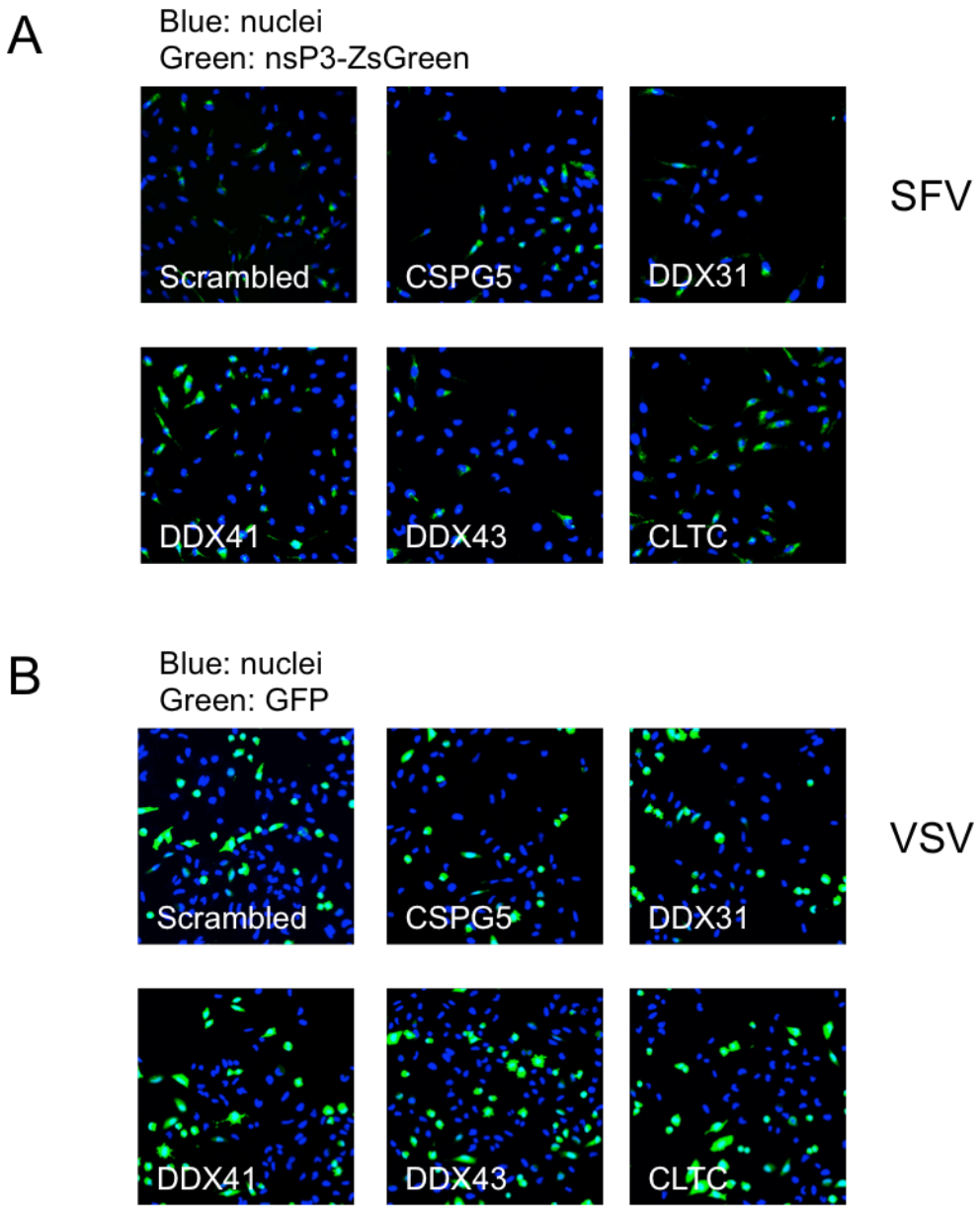

Figure 11. Representative images of cells depleted of host factors that do not affect SFV of VSV infections. A: HeLa cells infected with SFV-ZsGreen. Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images. **B:** HeLa cells infected with VSV-GFP. Nuclei are shown in blue and GFP, indicating VSV infection, is shown in green. The siRNAs used are indicated by the text in the images

4.2 Low pH-Induced PM fusion can be used to bypass the normal entry and penetration steps of SFV infection

To determine if depleted host factors play a part in the early stages (entry or penetration) of SFV infection, these steps were circumvented by artificially inducing viral envelope fusion at the PM (White *et al*, 1980). This was achieved by allowing the viruses to bind cells on ice and treating the cells quickly with acidic medium (pH 5.5). This causes a conformational change in the SFV spike

proteins, which leads to membrane fusion, delivery of nucleocapsid into the cytoplasm, and uncoating of the nucleocapsid. Since within the 90 second of low-pH treatment, some SFV virions are internalized by endocytosis, after the acid treatment cells were incubated in NH_4Cl-containing medium at pH 7.2 which neutralized the acidic pH of endosomes preventing these viruses from fusing (Figure 12). Compared to the normal route of SFV infection, the endocytic bypass was slightly less efficient, restoring about 60 % of infected cells. If the cells were not treated with acidic medium, but incubated in NH_4Cl-containing medium, the infection percentage dropped to the level of non-infected wells (Figure 13).

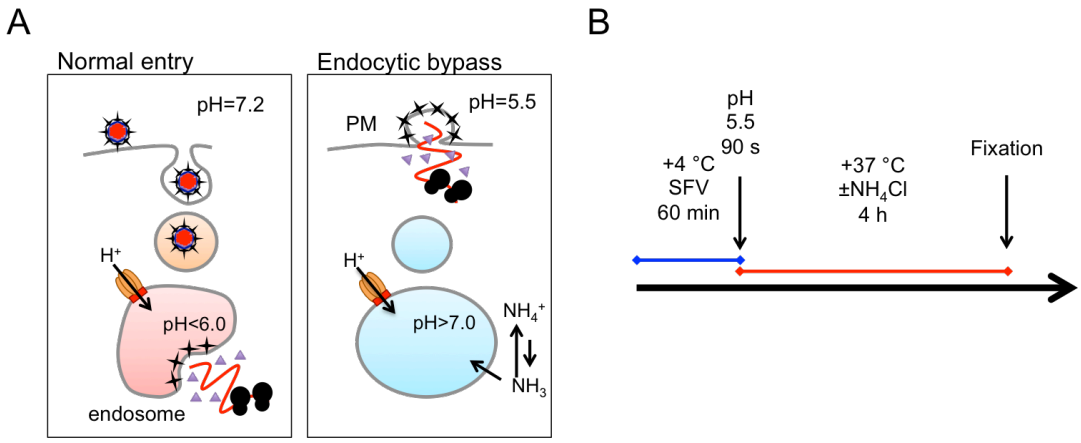

Figure 12. Endocytic bypass assay. A: Normally SFV binds to the plasma membrane and is internalized using receptor-mediated endocytosis. The endosome progressively acidifies and a pH lower than 6.0 causes a conformational change in SFV spike proteins, which triggers membrane fusion between SFV envelope and the endosomal membrane, freeing the nucleocapsid into the cytosol. The endosomal pathway can be bypassed by allowing SFV virions to bind to the host PM in the cold, and subsequently treating the infected cells with acidic medium for a short time. This leads to membrane fusion, and the release of the SFV nucleocapsid into the cytoplasm. To prevent endocytosed viruses from completing their infection through the endosomal route, the acidic medium is replaced with a medium containing NH_4Cl. Some of the NH_4^+ ions are converted into NH_3, a gas that can freely diffuse into the endosome and neutralize its acidification (White *et al*, 1980). **B**: In the endocytic bypass assay, SFV virions are allowed to bind host cells on ice for 60 minutes and the virus-containing medium is removed. Cells are treated briefly (90 s) with acidic medium, which it is then replaced with growth medium (pH 7.2) with or without NH_4Cl. Cells are incubated for 4 h at +37 °C and then fixed using 4% PFA.

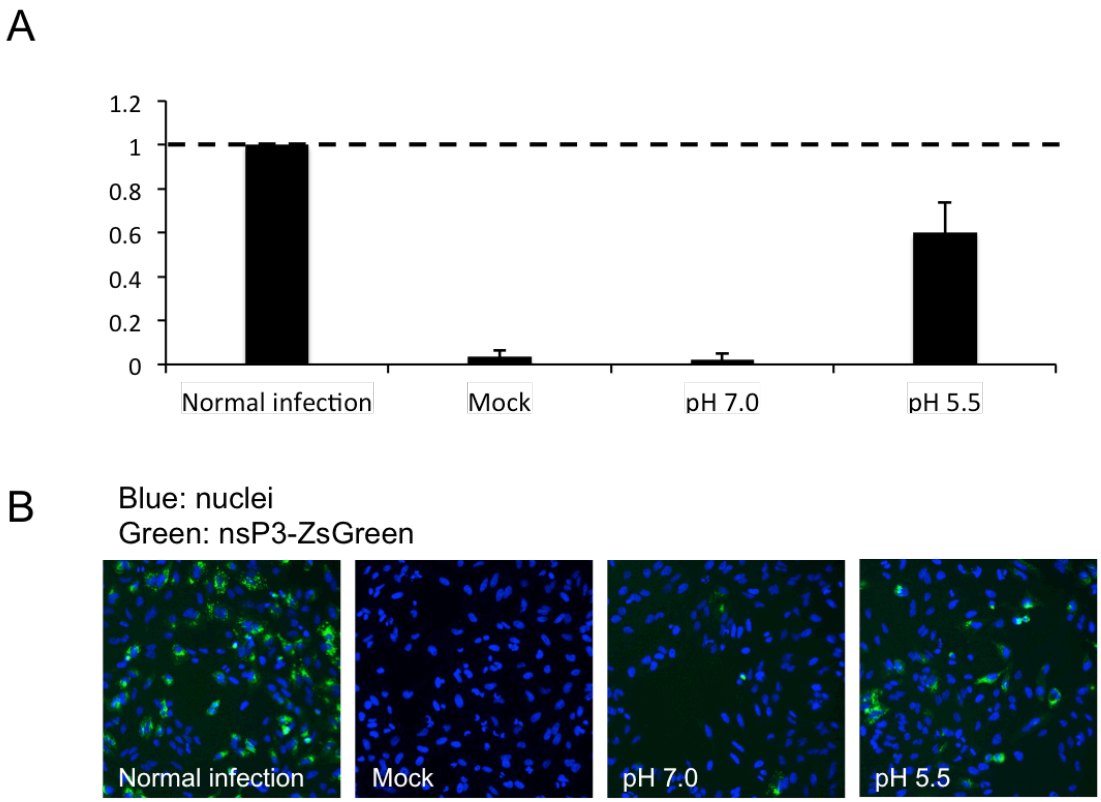

Figure 13. HeLa cells can be infected by using endocytic bypass. A: The relative infection percentages of HeLa cells infected with SFV-ZsGreen from two repetitions are indicated on the y-axis. The indicated pH values refer to infection with no fusion on the PM but incubation with NH_4Cl-containing medium (pH 7.0) and infection with PM fusion and incubation with NH_4Cl-containing medium (pH 5.5). The infection percentages were normalized by setting the mean of the normal infections as 1. The error bars represent standard deviations of the two repetitions. **B:** Representative images of HeLa cells infected with SFV-ZsGreen. Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The text in the images indicates different infection conditions (see A).

4.3 SLC6A13 is needed in the early stages of SFV infection

SLC6A13, also known as GAT-2, is a GABA and taurine transporter that contains 12 hydrophobic membrane-spanning domains, 11 loop regions and cytoplasmic N and C termini (Kristensen *et al*, 2011; Zhou *et al*, 2012). On the 3–4 loop there are three N-glycosylated residues (Figure 14) (Kristensen *et al*, 2011). This PM protein belongs to the solute-carrier 6 (SLC6) gene family, which contains membrane proteins that transport neurotransmitters by utilizing Na^+ and Cl^- ions (Kristensen *et al*, 2011). As it seems to be required for SFV infection, I tested if it is needed for entry and penetration steps of infection. By infecting HeLa cells using endocytotic bypass, I could revert SFV infectivity back to the level observed in the scrambled controls (Figure 15). For further controls I used siRNAs against ATP6V1B2, ATP6V1G1, and UPF1. ATP6V1B2 and ATP6V1G1 are known to be critical for the penetration of SFV, and UPF1 works against SFV

infection in the post-penetration stages (Balistreri *et al*, 2014). Endocytic bypass assay counteracted the effect of the siRNAs against ATP6V1B2 and ATP6V1G1 (Figure 15). Due to high variation, the SFV infectivity on UPF1-depleted cells was not statistically different from controls in the endocytic bypass. However, the mean infectivity was similar to the normal infection assay (1.5-fold higher than in controls) (Figure 15). Additionally, I used endocytic bypass to pinpoint whether DDX54, an RNA helicase, affects the entry and penetration, or post-penetration stages of SFV infection. The SFV infection in DDX54-depleted cells did not revert to the level of the controls when using the endocytic bypass assay (Figure 15). The ATP6V1B2, ATP6V1G1, and UPF1 bypass results, combined with the fact that the infectivity in DDX54-depleted cells remained significantly lower than in controls show that endocytic bypass does not revert the effects of gene knockdown in an unspecific manner. Therefore, it can be used to distinguish the host factors that are needed for the entry and penetration from the ones needed in the post-penetration stages.

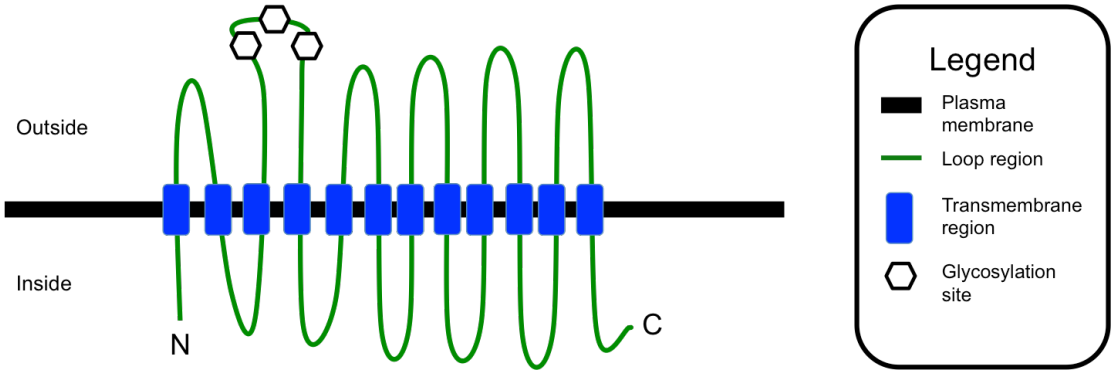

Figure 14. A schematic view of SLC6A13. SLC6A13 is a membrane protein that forms a transport channel for GABA and taurine at the PM (adapted from Kristensen *et al*, 2011).

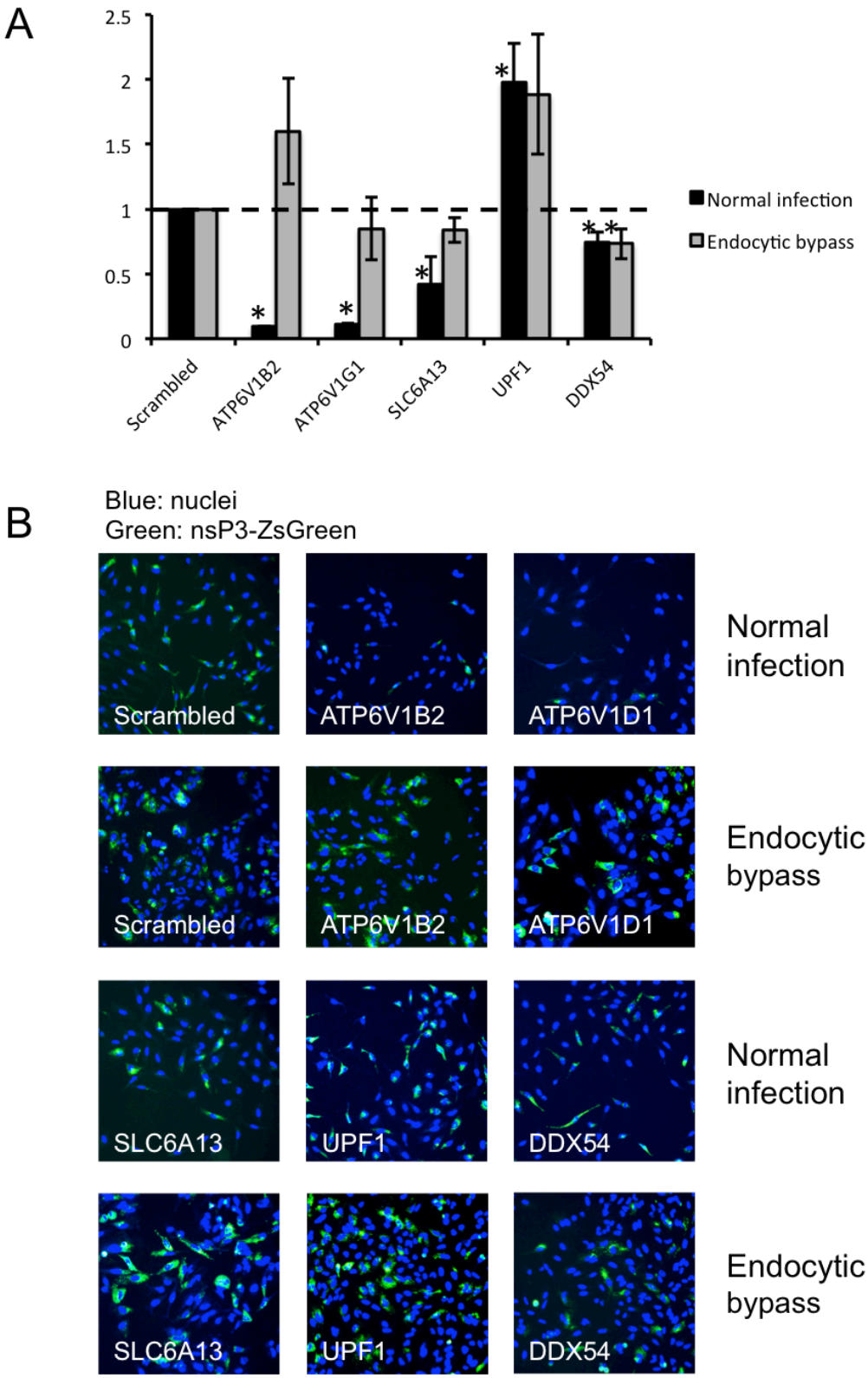

Figure 15. The roles of SLCA13 and DDX54 in SFV infection. A: The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen normally (black) or using endocytic bypass (grey) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control. **B:** HeLa cells infected with SFV-ZsGreen normally or using endocytic bypass (indicated on the right). Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images.

4.4 TNP01 and HDAC6 affect the entry and penetration steps of SFV infection.

TNP01 is a cellular transport molecule implicated in nuclear transport, mitotic spindle assembly, nuclear envelope assembly, ciliary import, and the formation of P bodies and stress granules (Twyffels *et al*, 2014). HDAC6 is member of the histone deacetylase (HDAC) family, but unlike other HDACs, it has also non-histonic targets and has a ubiquitin-binding zinc-finger domain (Li *et al*, 2013). Both TNP01 and HDAC6 have roles in cellular stress responses (Kawaguchi *et al*, 2003; Twyffels *et al*, 2014) and seem to have role in SFV infection, even though the effect of HDAC6 is minuscule. In the endocytic bypass assay, SFV infectivity was not significantly different from the controls in both TNP01 and HDAC6-depleted cells (Figure 16). This indicates that their roles are related to the early stages of SFV infection.

4.5 Components of the translation machinery affect penetration and post-penetration steps of SFV infection

EIF2B3, EIF4G1, and RPL18 are components of the translational machinery (Walsh & Mohr, 2011; de la Cruz *et al*, 2015) (Figure 17), and they appear to play a part in SFV infection. As their canonical roles are related to host translation, I assumed that they would work similarly in SFV infection and affect the post-penetration stages. I tested this using the endocytic bypass assay. This approach revealed that EIF2B3 is not needed in SFV entry or penetration, as the percentage of infected cells was three times that of controls in the endocytic bypass assay (Figure 18). For the EIF4G1-depleted cells, the mean infection percentage in the endocytic bypass assay was similar to that of normal infection assay (1.5-fold higher than in controls), but this difference was not statistically significant (Figure 18). Surprisingly, by circumventing endocytosis, I could fully revert the infection-diminishing effect of the RPL18 knockdown (Figure 18), indicating that this ribosomal subunit plays a part in the early stages of SFV infection.

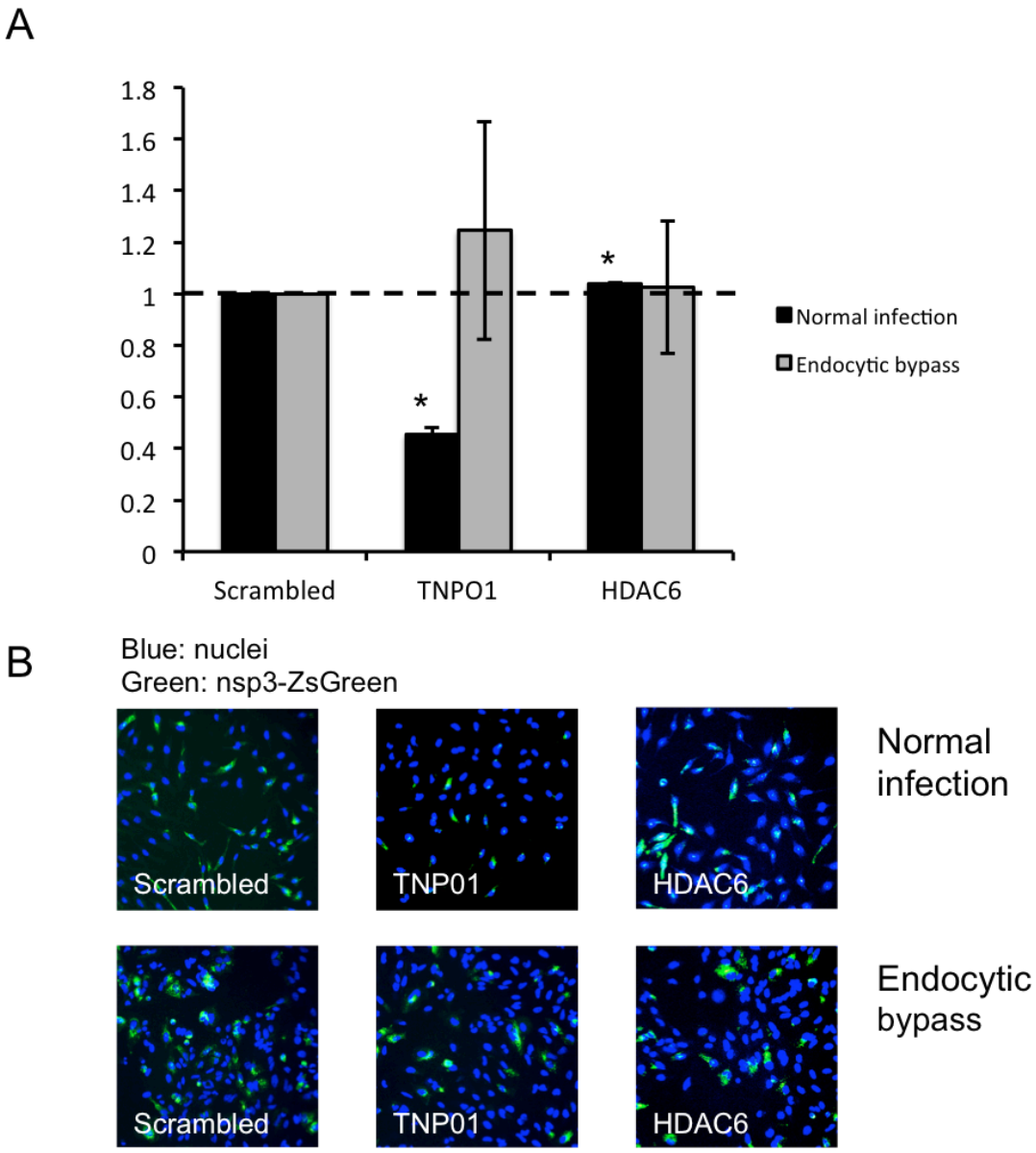

Figure 16. The roles of TNP01 and HDAC6 in SFV infection. A: The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen normally (black) or using endocytic bypass (grey) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control. **B:** HeLa cells infected with SFV-ZsGreen normally or using endocytic bypass (indicated on the right). Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images.

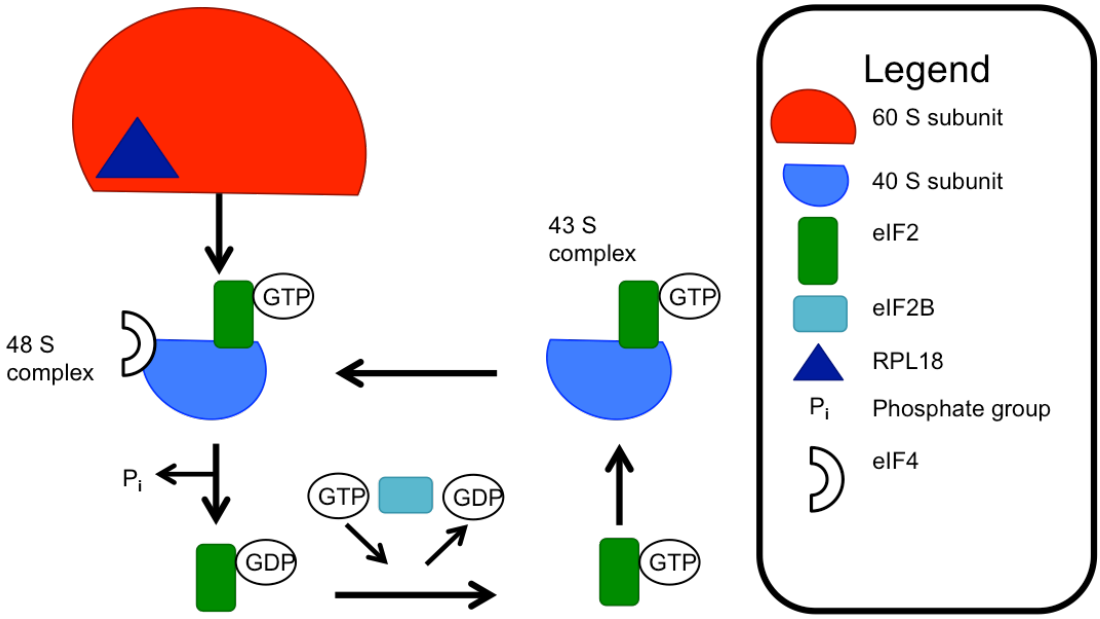

Figure 17. The roles of eIF2B, eIF4, and RPL18 on the initiation of translation. During the initiation of eukaryotic translation, a 43 S pre-initiation complex is formed by GTP-bound eIF2, the 40 S ribosomal subunit and other translation factors (Walsh & Mohr, 2011). This complex binds eIF4 and other translation factors, forming the 48 S pre-initiation complex (Walsh & Mohr, 2011). Following GTP hydrolysis and the binding of the 60 S subunit, GDP-bound eIF2 dissociates and translation moves to the elongation phase (Walsh & Mohr, 2011). GDP-eIF2 is converted to active GTP-eIF2 by eIF2B and it can initiate translation again (Walsh & Mohr, 2011). RPL18 is a part of the ribosomal large subunit (de la Cruz *et al*, 2015). Most translation factors and mRNA are omitted for clarity.

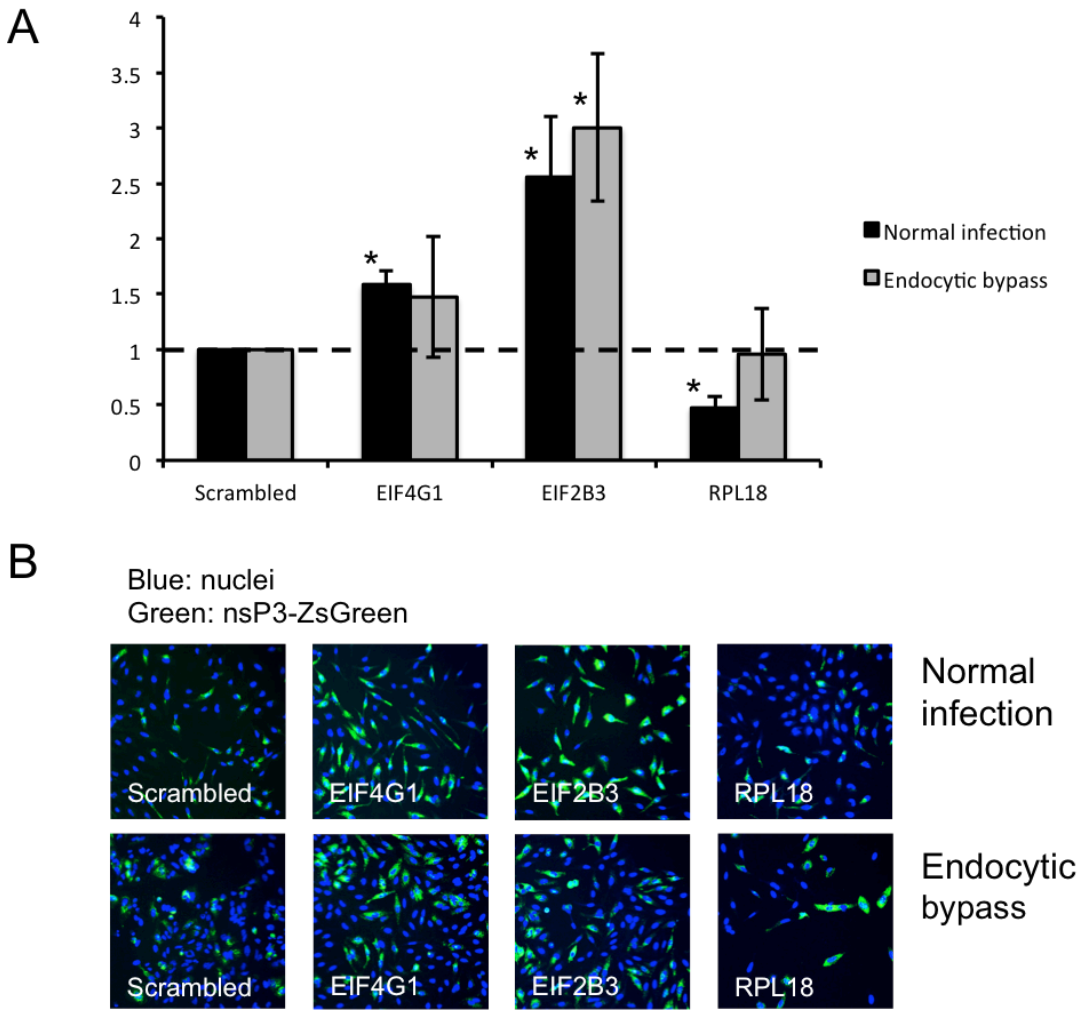

Figure 18. The roles of different translational machinery components in SFV infection. A: The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen normally (black) or using endocytic bypass (grey) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control. **B:** HeLa cells infected with SFV-ZsGreen normally or using endocytic bypass (indicated on the right). Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images.

4.6 SFV infection is affected by other host factors in penetration and post-penetration steps

SFV infectivity could be increased by using siRNAs against PHB2, EDF1, ETF1, DDX47, DHX37, and DHX57. DDX47 and DHX57 are probable RNA helicases (Gene IDs: 51202 and 90957, respectively), DDX54 is a RNA helicase that functions in transcription regulation (Rajendran *et al*, 2003; Kanno *et al*, 2012), PHB2 and EDF1 are implicated in transcriptional processes (Kabe *et al*, 1999; Bavelloni *et al*, 2015), and ETF1 is known to interact with nonsense-mediated decay (Czaplinski *et al*, 1998), which is a part of the host antiviral defense

(Balistreri *et al*, 2014). Therefore my hypothesis was that all of these factors would have post-penetrational effects. The effects of PHB2 and EDF1 depletion were similar in the endocytic bypass and normal infection (Figure 19) so I concluded that their effects are not related to SFV entry or penetration. In ETF1, DDX47 and DHX37-depleted cells the infectivity was not statistically significantly different from the controls, but for DDX47 and DHX37 it was still high and comparable to the infectivity in the normal infection assay (2-fold and 1.5-fold higher than controls, respectively) (Figure 19). Curiously, in the DHX57-depleted cells, the effect of the endocytic bypass was the opposite of the normal infection, as the infectivity was significantly lower than in controls (opposed to higher than in controls in the normal infection).

The depletion of GNPDA1 and DMN2 decreased SFV infectivity. As DMN2 has a crucial role in endocytosis (Kasai *et al*, 1999), I assumed that effect of knocking it down would be limited to the early stages of SFV infection. GNPDA1, on the other hand, is a poorly-characterized glucosamine-6-phosphate deaminase (Wolosker *et al*, 1998) so it was difficult to hypothesize how it affects SFV infection. By using endocytic bypass assay, the effect of the siRNA treatments against GNPDA1 and DMN2 were reversed, so it seems that they both have roles in the early stages of SFV infection (Figure 19).

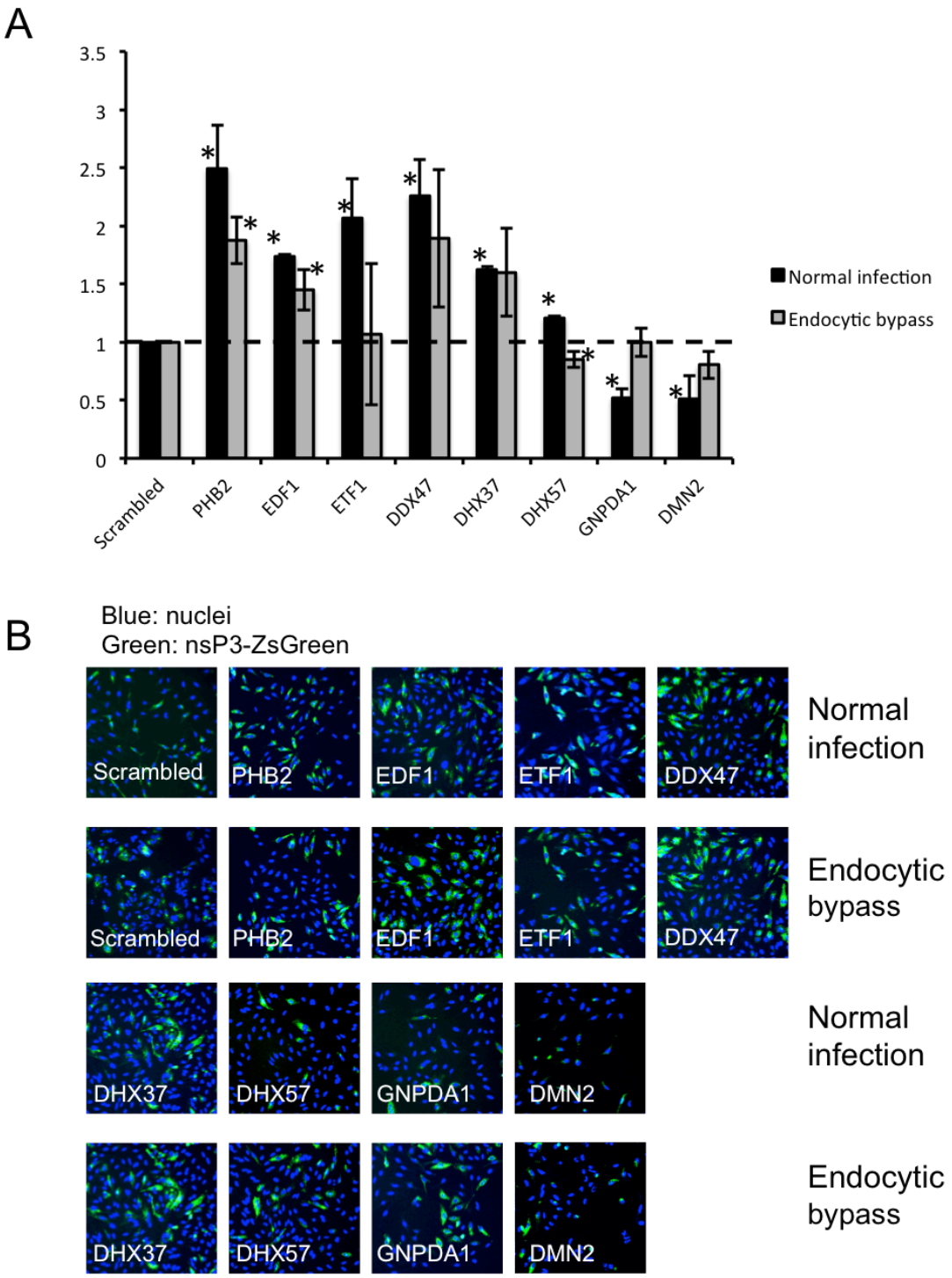

Figure 19. The roles of PHB2, EDF1, ETF1, DDX47, DHX37, DHX57, GNPDA1, and DMN2 in SFV infection. A: The relative infection percentages of siRNA-treated HeLa cells infected with SFV-ZsGreen normally (black) or using endocytic bypass (grey) are indicated on the y-axis. The siRNAs used are indicated on the x-axis. The infection percentages were normalized by setting the mean of the scrambled controls as 1. The error bars represent standard deviations of the tree repetitions and an asterisk signifies a result that is significantly different from the control. **B:** HeLa cells infected with SFV-ZsGreen normally or using endocytic bypass (indicated on the right). Nuclei are shown in blue and nsP3-ZsGreen, indicating SFV infection, is shown in green. The siRNAs used are indicated by the text in the images.

5. Discussion

5.1. Host factors in SFV and VSV infections

Using siRNA knockdown, high-throughput imaging, and automated image analysis, I studied the effects of the depletion of various host factors on SFV and VSV infection (Table 2).

Table 2. The effects of siRNA treatment against various genes on SFV and VSV infection

Gene	Effect on SFV infection*	Effect on VSV infection*	Gene	Effect on SFV infection*	Effect on VSV infection*
PHB2	+	-	DDX41	0	0
EDF1	+	0	DDX43	0	0
SLC6A13	-		DDX47	+	0
EIF2B3	+	0	DDX54	-	0
ETF1	+	0	DHX37	+	-
EIF4G1	+	0	DHX57	+	0
DDX18	0	-	KREMEN2	0	+
PVR	0	+	HDAC6	+	0
CSPG5	0	0	DNM2	-	0
GNPDA1	-	0	AP2M1	0	+
RPL18	-	-	CLTC	0	0
UPF1	+	0	KPNB1	0	-
ATP6V1B2	-	-	TNPO1	-	+
ATP6V1G1	-	-	Ppbi	0	+
DDX31	0	0	PPBI	0	+

* + = siRNA treatment increases infection, - = siRNA treatment decreases infection, 0 = siRNA treatment has no effect on infection

By using the endocytic bypass assay, I pinpointed the roles of SLC6A13, TNP01, HDAC6, RPL18, ETF1, and GNDPA1 to the early events in SFV infection. I also employed the same method to assign post-penetration roles for DDX54, EIF2B3, PHB2, and EDF1. My results also confirmed the previously reported roles of ATP6V1B2 and ATP6V1G1 in the early stages of SFV infection (Balistreri *et al*, 2014). In the case of EIF4G1, DDX47, and DHX37 the mean infectivity in the endocytic bypass was similar to normal infection, even though the difference was not statistically significant. In all cases this was caused by a single outlying

repetition (data not shown), so I am inclined to believe that these factors affect the later stages of SFV infection. Similarly, in the endocytic bypass assay the mean infectivity in UPF1-depleted cells was not significantly different form the control because of a single outlier (data not shown). Still, the mean infectivity was similar to the normal infection, and almost two times that of the controls. Furthermore, UPF1 has been reported to prevent the translational and transcriptional stages of SFV infection (Balistreri *et al*, 2014). Curiously, in the normal infection, DHX57 depletion increased SFV infection significantly but in the endocytic bypass, virus infectivity was significantly lower than in controls.

5.2. SLC6A13 is a candidate receptor for SFV

SLC6A13 is structurally similar to the iron transporter NRAMP2 (natural resistance associated macrophage protein 2) (Nevo & Nelson, 2006; Kristensen *et al*, 2011). They both belong to the same family of solute-carrying membrane proteins that have 12 membrane-spanning domains and cytosolic N- and C-termini (Nevo & Nelson, 2006; Kristensen *et al*, 2011). NRAMP2 is used as a receptor in both insect and mammalian cells by Sindbis virus, an alphavirus that is closely related to SFV (Rose *et al*, 2012). Therefore SLC6A13 might be the receptor for SFV. This is further supported by the fact that SLC6A13 depletion does not affect VSV infectivity, as receptors are often virus-specific (Grove & Marsh, 2011). Additionally, SLC6A13 mRNA is transcribed heavily in the kidneys (The Human Protein Atlas, http://www.proteinatlas.org/ENSG00000010379-SLC6A13/tissue, Fagerberg *et al*, 2014). The currently used SFV strains have been passaged multiple times in hamster kidney (BHK-21) cells (Atkins *et al*, 1999) and the reason that BHK-21 cells support high titers of SFV may be the presence of the hamster analogue of SLC6A13. Passaging has affected SFV binding by selecting for single amino acid mutations in the envelope proteins of the virus which allows SFV to bind to host heparan sulphate via simple electrostatic interactions (Smit *et al*, 2002). However binding to host receptors is more complicated than binding on the surface, as it involves multiple specific interactions between viral and host proteins (Marsh & Helenius, 2006). These events need to be complex enough signal the host to initiate the endocytic program (Marsh & Helenius, 2006). It is therefore conceivable that adapting to

completely new receptors is an unlikely event even in cell culture, and viruses may retain specificity for suitable receptors on the surface of the cells from which they are produced.

To confirm the role of SLC6A13 in SFV infection, further experiments are needed. We will test if SLC6A13-depletion blocks SFV entry (as opposed to penetration) using single-virus tracking in living cells and (Hoornweg *et al*, 2016). We will also use fluorescent immunolabeling and confocal microscopy to compare the rate of SFV endocytosis in SLC6A13-depleted and wild type cells (Rizopoulos *et al*, 2015). Furthermore, will determine, if the transfection of a SLC6A13-producing plasmid will rescue SFV infection in conditional SLC6A13-knockout cells. To further characterize the potential interaction between SLC6A13 and SFV, a structural approach is needed. This may, for example, be in the form of X-ray crystallography of the SFV spike proteins complexed with a SLC6A13 extracellular domain or domains (Peng *et al*, 2011), or a cryo-electron microscopy single-particle reconstruction of SFV bound to SLC6A13 (He *et al*, 2002).

5.3. Host factors that affect SFV infection

The fact that TNP01 RPL18, and ETF1 are needed for SFV entry and penetration stages is surprising as TNP01 is involved in intracellular trafficking and RPL18 and ETF1 have canonical roles in translation (Taylor *et al*, 2012; Twyffels *et al*, 2014; de la Cruz *et al*, 2015). TNP01 is used by adenoviruses (Hindley *et al*, 2007), human immunodeficiency virus 1 (HIV-1) (Arnold *et al*, 2006), and human papillomavirus (Darshan *et al*, 2004) for the nuclear transport of various virus components. As the effect of TNP01 depletions are reversed by the endocytic bypass assay, this cellular protein must be needed in the very early stages of SFV infection. Therefore, a role in the later steps of the virus life cycle, such as interference with stress granule formation or TNP01-mediated nuclear import of viral replicase protein nsP2 (Twyffels *et al*, 2014; Fros & Pijlman, 2016), are unlikely to be related to the observed phenotypes. It might also be that the effect of TNP01 depletion is indirect and it affects the function of some other proteins that have roles in SFV infection.

In the case of VSV, the known role of TNP01 in stress granule formation, a process that is considered as a mechanism of intrinsic antiviral immunity may be the reason why TNP01 depletion increases VSV infection (Beckham & Parker, 2008; Twyffels *et al*, 2014). It has previously been reported that the knockout of TIA-1, a stress granule-associated protein, increases the infectivity of VSV in mouse embryonic fibroblasts (Li *et al*, 2002), which further supports this hypothesis.

Based on the interaction of RPL18 and other viruses, one would expect RPL18 to affect the late stages of SFV infection. This ribosomal protein has a role in the translation or replication of Dengue virus (Cervantes-Salazar *et al*, 2015), cauliflower mosaic virus (Leh *et al*, 2000) and HCV (Dhar *et al*, 2006). However, in SFV infection, RPL18 functions in the early stages of the viral life cycle, which indicates a mechanism different from the other known viruses. It has been shown that ribosomes are needed in the uncoating of SFV nucleocapsids (Singh & Helenius, 1992). Thus, RPL18 might have a role in this process, which would be a function unlike any described for a ribosomal protein so far. However, this would also mean that during the endocytic bypass assay, when the nucleocapsids are delivered directly to the cytoplasm through the PM, SFV virions are uncoated in a different way than during the normal entry. This unexpected result may also be caused by an indirect interaction of RPL18 and some other cellular factor, which is perturbed by the depletion of RPL18. As I did not investigate at which stage of VSV infection RPL18 affects, my working hypothesis is that it probably affects the replication or translation, as has been reported for other viruses (Leh *et al*, 2000; Dhar *et al*, 2006; Cervantes-Salazar *et al*, 2015).

ETF1 is another protein that seemed to be related to the post-entry stages of SFV infection. ETF1 is a eukaryotic translation termination factor, with a role in nonsense-mediated decay (Czaplinski *et al*, 1998), a pathway that is known to counteract SFV infection during translation and replication (Balistreri *et al*, 2014). Thus, I initially assumed that ETF1 would have a role in the post-penetration stages of SFV infection. My results rule out this possibility, because the effect of ETF1 depletion was reversed by the endocytic bypass assay.

Therefore, it might be that the effect I have observed is indirect, or ETF1 has previously uncharacterized functions. Additionally, these functions do not seem to be broadly antiviral, as ETF1-depletion had no effect on VSV infection.

HDAC6 is used by influenza A virus (IAV) to uncoat its genome (Banerjee *et al*, 2014). It is also reported to inhibit oncolytic herpes simplex virus infection in glioma cells, apparently by interfering with endocytic trafficking (Nakashima *et al*, 2015). As our initial result showed small but reproducible increase in SFV infection followed by HDAC6 knockdown, I assumed that it plays a minor role in SFV entry and penetration processes as HDAC6 has a role in the positioning of endosomes (Li *et al*, 2013). This was confirmed by endocytic bypass. Surprisingly HDAC6 depletion did not affect VSV infection, even though HDAC6 has been reported to protect mice from VSV (Choi *et al*, 2016). This may indicate that cell culture differs significantly from *in vivo* conditions or that HDAC6 has different roles in mouse and human cells. It is also possible that HDAC6 functions in the very late stages of VSV infection, such as egress, as our assay only quantifies viral replication and not the later stages of infection.

DMN2 is another protein with a function in endocytosis (Kasai *et al*, 1999), and therefore it was expected that its depletion would reduce SFV infectivity by affecting the early steps of the infection. The role of DMN2 in virus infection is well characterized, and it is needed for example by bovine ephemeral virus, hepatitis E virus, and Pichindé virus (Vela *et al*, 2008; Cheng *et al*, 2012; Holla *et al*, 2015). Surprisingly it was not required by VSV, even though it is reported to be crucial for VSV entry (Cureton *et al*, 2009; Johannsdottir *et al*, 2009).

In cells, EIF2B3 and EIF4G1 function in transcription initiation (Walsh & Mohr, 2011). EIF2B3 is a component of eIF2B, which recycles eIF2, a key factor in the formation of the 43 S pre-initiation complex and EIF4G1 has a function in bringing the mRNA cap and poly-A tail together in the 48 S pre-initiation complex (Walsh & Mohr, 2011). As a part of the host's broad antiviral response that aims to shut down translation, eIF2 can be inactivated by phosphorylation (Walsh & Mohr, 2011). SFV can counteract this response, and is known to be able

to translate its structural genes even in the presence of phosphorylated eIF2. As the depletion of EIF2B3 increases SFV infection in the post-penetration stages, it seems that SFV can translate even its early genes without a canonical set of translation initiation factors. Knocking down EIF4G1 similarly affects later stages of SFV infection and increases SFV infectivity. This shows that though cap-dependent, the translation of SFV early genes does not occur in exactly the same way as the translation of host mRNAs. Since SFV RNA is not as susceptible to changes in the composition of the translation initiation complex as host mRNAs are, it gains an advantage in the translational competition. As the knockdown of EIF4G1 or EIF2B3 does not affect VSV infection, their function is most likely not broadly antiviral. This indicates that SFV has evolved some mechanism to benefit from the depletion of these host factors while VSV has not. Most likely this is difference is mediated by the differences in SFV (sub)genomic RNA and VSV mRNAs.

PHB2 knockdown increased SFV infectivity during the post-penetration steps and it also increased VSV infectivity. PHB2 has been reported to be a pro-viral agent for HIV-1 (Emerson *et al*, 2010) and to associate with severe acute respiratory syndrome coronavirus nsPs (Cornillez-Ty *et al*, 2009). Curiously, PHB2-depletion has been previously shown to reduce VSV infection in mouse cells (Kreit *et al*, 2015). The different result reported here might be due to different roles of PHB2 in murine and human cells, or differences in VSV infection between these two organisms. Additionally, Kreit *et al* (2015) used small hairpin RNA technology, opposed to siRNAs used in this study. Even though these methods are highly similar, this may have a role in the differing results. As PHB2 interacts with a myriad of host processes and proteins (Bavelloni *et al*, 2015), it is possible that the effects of its depletion are indirect and the actual effects are mediated by other host factors. PHB2 is also a transcriptional regulator (Bavelloni *et al*, 2015) so its knockdown may just reduce host translation, thus giving the viral RNAs an edge in the translational competition. This may also be the mechanism by which EDF1-depletion promotes both SFV and VSV infectivity, as it is a transcriptional activator (Kabe

et al, 1999). This is further supported by the fact that the effect of EDF1 knockdown affects the later stages of SFV infection.

GNDPA1, DDX54, DDX47, DHX37, and DHX57 are all poorly characterized cellular factors that have not been previously implicated in viral infections. DDX47, DHX37, and DHX57 are predicted RNA helicases (Gene IDs: 51202, 57647, and 90957, respectively). DDX54 is an RNA-helicase that has been reported to be a transcriptional co-repressor (Rajendran *et al*, 2003; Kanno *et al*, 2012). The knockdown of DDX53, and DDX47 increased SFV infection by affecting the post-penetration stages, but had no effect on VSV infection. This may indicate that both of these helicases can target RNA structures found in SFV but not in VSV. Or possibly SFV but not VSV requires these helicases for translation or transcription. DHX37 depletion affected both SFV and VSV infections. It decreased SFV infection, by affecting the late stages, and increased VSV infection. This may be due to a similar situation as above, but with DHX37 depletion affecting host RNAs in a way, that allows VSV to succeed in the translational competition against the host. It is difficult to hypothesize the reason why DHX57 depletion was beneficial to SFV in the normal infection, but detrimental in the endocytic bypass. This may be due to a complex role of DHX57 or merely experimental anomalies. GNDPA1 is a highly conserved protein that plays a crucial role in metabolism (Wolosker *et al*, 1998). It is, therefore, surprising that it would have a role in the early stages of SFV infection. This effect would imply that GNDPA1 has previously uncharacterized functions, or that its depletion affects other host factors that, in turn, affect SFV infection.

5.4. Other host factors

Apparently, VSV infection is counteracted by PVR, KREMEN2, AP2M1 and cyclophilin B. On the other hand VSV seems to need ATP6V1B2, ATP6V1G1, DDX18, and KPNB1 for successful infection. The requirement of the vATPase subunits ATP6V1B2 and ATP6V1G1 by VSV is not surprising, as it penetrates host membranes via pH-dependent fusion (Regan & Whittaker, 2013). PVR and KREMEN2 are cell surface molecules (Mendelsohn *et al*, 1989; Mao *et al*, 2002) and AP2M1 is a part of AP2, and therefore has a role in clathrin-mediated

endocytosis (Heinaman, 1995; Boucrot *et al*, 2010). Therefore it is conceivable that these molecules would affect VSV entry or penetration.

DDX18 is a poorly-studied putative RNA helicase for which helicase activity has not been demonstrated yet (Dubaele & Chène, 2007). If DDX18 does affect RNA, the effect of its depletion on VSV but not SFV infection may be due to VSV containing some RNA motif that SFV lacks, or vice versa. KPNB1 is a protein required for nuclear import (Görlich *et al*, 1995) and its depletion greatly reduces VSV infection. It has been previously shown that the M protein of VSV is transported into the nucleus, which allows it to reduce nucleocytoplasmic transport (Petersen *et al*, 2000; Glodowski *et al*, 2002). Perhaps the transport of the M protein is mediated by KPNB1, which reduces the transport of host mRNAs into the nucleus, therefore giving VSV the upper hand in the translational competition.

Cyclophilins affect the infections of multiple viruses (Frausto *et al*, 2013) and cyclophilin A has been reported to be required by VSV for successful infection (Bose *et al*, 2003). The siRNAs against PPBI increased infectivity more than the siRNAs against Ppbi. Most likely this effect is explained by sequence dissimilarity between human and mouse cyclophilin B as the use of Ppbi-targeting siRNAs would result in only partial knockdown of PPBI (Petersen *et al*, 2000; Glodowski *et al*, 2002).

CSPG6, a chondroitin sulfate proteoglycan (Watanabe *et al*, 1995), DDX31, an RNA-helicase with a possible role in renal carcinogenesis (Fukawa *et al*, 2012), DDX41 a RNA helicase with functions in immune processes (Jiang *et al*, 2017), DDX43, an RNA and DNA helicase (Tanu *et al*, 2017), and CLTC, a well-characterized endocytic protein (Doherty & McMahon, 2009) did not have roles in SFV of VSV infections. Chondroitin sulfate proteoglycans have been implicated in the binding of viruses to host cells (Banfield *et al*, 1995; Hsiao *et al*, 1999), but SFV and VSV apparently do not utilize it, at least exclusively. This is consistent with the report that SFV binds to heparan sulfate on the host surface (Smit *et al*, 2002). An siRNA screen showed a reduction in the infection of IAV (a double-

stranded DNA virus) followed by the silencing of DDX31, but the investigators did not pursue this result any further (Diot *et al*, 2016) and DDX41 seems to have a role in innate immunity in the detection of double-stranded DNA viruses (Jiang *et al*, 2017). As SFV and VSV are RNA viruses, it seems that virus-affecting roles of DDX31 and DDX41 might be exclusive to DNA viruses. DDX43 has not been previously implicated in viral infection so it is not surprising that it does not affect SFV or VSV infections. Clathrin has been thought to be crucial for the entry of both SFV and VSV (Helenius *et al*, 1980; Sun *et al*, 2005), but according to my results the entry process might be more complicated, and CLTC is not required by either virus.

5.5. Methodological considerations

As siRNA-based screening has become cheaper and siRNA design has improved, a number of large-scale studies on host-virus interactions have been published in recent years (Perreira *et al*, 2016). Even though siRNA-based screening is definitely a powerful tool, it has its drawbacks. Traditionally, siRNAs have been considered very specific but Franceschini *et al* (2014) showed that siRNAs used in screening can act as micro-RNAs (miRNAs) and have strong off-target effects. This kind of indirect effects may explain the surprising results of siRNA treatments against TNP01, RPL18, and ETF1 on SFV infection as well as those of siRNAs against PHB2 and HDAC6 on VSV infection. The apparent nonessentiality of CLTC in both SFV and VSV infections may also be explained with off-target effects. It may also be, that some of the surprising results are due to incomplete knockdown of the studied host factors. Due to possibility of off-targets or incomplete knockdown, the results of siRNA screens need to be extensively validated. The knockdown efficiency of the siRNAs should be tested using immunoblotting. Preferably, the roles of the genes of interest would be studied using knockout cells. As an ultimate test, a rescue assay needs performed, in which knockdown or (preferably) knockout cells are transfected with a plasmid that produces the protein in question. This should lead to the reversion of the effect on virus infection. Due to the lack of further validation, the genes implicated by my experiments are to be understood as candidate factors.

The endocytic bypass assay used in my experiments is very robust and separates the function of the host factors to two rather broad categories. Therefore, additional experiments are needed to perfectly understand the role of host factors. Using confocal microscopy, it is possible to detect if viruses bound on cell surface are endocytosed or not (Rizopoulos *et al*, 2015). To determine if the depletion of the host factor affects SFV penetration or uncoating, specific antibodies against the acidified spike proteins and SFV capsid proteins are available (Liao & Kielian, 2006) Spike acidification is a sign of membrane penetration, and the localization of the signal from anti-capsid antibody can be used to detect the uncoating events (Singh & Helenius, 1992). To pinpoint the effects of host factors to later stages of SFV infection, immunoblotting can be used to detect the production of early and late viral proteins. Similarly to SFV, the endocytic part of VSV infection can be bypassed (Blumenthal *et al*, 1987). Therefore, performing an endocytic bypass assay would be the next step in the elucidation of the roles of the host factors that affect VSV infection, followed with e. g. direct measuring of VSV endocytosis (Rizopoulos *et al*, 2015).

5.6. Conclusions

The major result of this study is that SLC6A13 is a candidate receptor for SFV and if validated, it implies that alphaviruses in general use structurally similar solute carriers as their receptors. This may lead to the discovery of the receptors, of other, more clinically relevant alphaviruses, which in turn may lead to new therapeutic applications. Overall, it is clear that multiple previously uncharacterized host factors affect both SFV and VSV infections and many of these factors are specific to SFV or VSV. This thesis also shows that host factors play important parts in both the early and late stages of SFV infection. Taken together, the results of this thesis further confirm that virus-host interactions are crucial in virus infections. My findings serve as a starting point to continue to in-depth studies into how these factors affect SFV and VSV infections.

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8. Supplementary information

Table S1. Sequences of the siRNAs

Gene symbol	Gene ID	siRNA sequences	Gene symbol	Gene ID	siRNA sequences
PHB2	11331	GUGAAUAUCUCCCUGCGAG CCACAUCACAGAAUCGUAU CAUCAAACUUCGCAAGAUU CAGAAUAUCUCCAAGACGA	DDX43	55510	CAACCAGAAUCAUUAGUCA CGGCGAAGCUCGACAGUGU CGCACAAUCUUAUUUGAAA GAGAGGAAGGUUUGAAAUG
EDF1	8721	UAUCUUAGCGGCACAGAGA UAACCAGGUGCUUGGCAAA CAGAGGAGCUGCACCAUGA CCACGAAAAUCAAUGAGAA	DDX47	51202	GGAUGAAGCCGACCGAAUA AGAAGAAACGCUCGCGAGA UCAAAGUGAUUCCUCGAGA CGAGUAGGUCGAACAGCUA
SLC6A13	6540	GCACAACCAGUAAUGGAGA GGGCACUGGAACAACAAGA AGAUCAUUGGCUUAGGCAA AAACCAGUGUAUCCAGUCA	DDX54	79039	GCACGAAAAUCCCGACAUA CGUAAGAAGAAGCGGUUUG GCAGAGGAGUUGCGGGUCU UGGAAUACGUGGUGUUCGA
EIF2B3	8891	GAAAAUGGGUCAAUAACUU AGACCUAACUUCCAGCAUU GGUGGAGGAUCUCGGAUGA GUUUAGAGCUUAUGAUGCA	DHX37	57647	CGGAUGGUGUGCUGCUUAA UCAAGAAACGCUACUACGA GAAAUGCGGAAGUUUAAGA CGACGACCCUGUCUUCAUC
ETF1	2107	GAGCUACGUUGGAAAUUGU UAACUAUGUUCGGAAAGUA AAACUGAACUAAGUCAAUC AACAUUAAGUCACGAGUAA	DHX57	90957	CAACAGACGUAGUAAUCGA ACAAGUAGAUUCCGCAAAU CUUCGGAACCUGUCGUAUA UCACCAAGAACGAUGGAUA
EIF4G1	1981	GGGCUUAGCUGGAAGGAAU GUUAAUGACCGAAGAUAUA GGGAACAGAAGUAUGAAUA GAUGUUAACAGAGGCAAUA	KREMEN2	79412	CGGACUUCCCGGACGAGUA GUCCAGGCCUGUCCGAAUG CAACACAGCUACAGCAGCG GAGCUCACCUUCCGCCUCU
DDX18	8886	GAUCUGAACGUCAACAGUA CGGAUGACCCUAAGGAAUA GAUAAUGGGUGGCAGUAAC GAAGUCGACUGGAUUGUUC	HDAC6	10013	GGGAGGUUCUUGUGAGAUC GGAGGGUCCUUAUCGUAGA GCAGUUAAAUGAAUUCCAU GUUCACAGCCUAGAAUAUA
PVR	5817	GGAUCGGGAUUUAUUUCUA CCAAACGGCUGGAAUUCGU GGGCAUGUCUCCUAUUCAG GCAAGAAUGUGACCUGCAA	DNM2	1785	GGCCCUACGUAGCAAACUA GAGAUCAGGUGGACACUCU CCGAAUCAAUCGCAUCUUC GAGCGAAUCGUCACCACUU
CSPG5	10675	CAGAUAUCAUUGACAUCGA UCGAAGGACUGGAUGGUGA GCGCAGAUCUGGGGAGCUU CCUUGGAGGUUUGGCUGAA	AP2M1	1173	GUUAAGCGGUCCAACAUUU GCGAGAGGGUAUCAAGUAU AGUUUGAGCUUAUGAGGUA GAACCGAAGCUGAACUACA
GNPDA1	10007	GAGACCACCCGGAGAGUUA GGAAUGCAGUCGACCUACA CAGGUGGGAUCGAGCUAUU UAUCACAGGUGCUCACAAG	CLTC	1213	GAGAAUGGCUGUACGUAAU UGAGAAAUGUAAUGCGAAU GCAGAAGAAUCAACGUUAU CGUAAGAAGGCUCGAGAGU
RPL18	6141	GGCUGUUGGUCAAGUUAUA AGCCCAAGAGCCAGGAUAU GAUCCUACUCUCUUAUUAA UAACAAGGACCGAAAGGUU	KPNB1	3837	GAACCAAGCUUGAUCUGUU GCUCAAACCACUAGUUAUA GACGAGAAGUCAAGAACUA GGGCGGAGAUCGAAGACUA
UPF1	5976	CAGCGGAUCGUGUGAAGAA CAAGGUCCCUGAUAAUUAU GCAGCCACAUUGUAAAUCA GCUCGCAGACUCUCACUUU	TNPO1	3842	GCAAAGAUGUACUCGUAAG GUAUAGAGAUGCAGCCUUA GUAAAUACCAGCAUAAGAA GCAAAUGUGUAUCGUGAUG
ATP6V1B2	526	CAGCUGAAUUUCUGGCGUA GCACUUAUGUUUACGGAUA UAUUCAAUGGAUCGGGAAA GGGAGAAACGGCUCGAUUA	Ppbi	19035	CAAGUUCCAUCGUGUCAUC CGGCAAAGUUCUAGAGGGC GGAGAAACCCUUCGCCAUU GAAAGAGCAUCUAUGGUGA
ATP6V1G1	9550	CAUGAAAACUACCGCAUAA CAGACAUACUUCCGGCAGA AUUACUUGGUGUAUCGAUA UUAGAUGCCCUCACGAAUA	PPBI	5479	ACAGCAAAUUCCAUCGUGU GAAAGAGCAUCUACGGUGA GAAAGGAUUUGGCUACAAA GGAAAGACUGUUCCAAAAA
DDX31	64794	CCAAAGAAGCAUUCGGUUA CCGUACAGCUGGAGCCGUU GCGAAACGGAGGAACGAAA GCAAGCAAAGGCUACGAAA	KIF11	3832	GAGCCCAGAUCAACCUUUA GGAGAGGUCUAAAGUGGAA UCGGGAAGCUGGAAAUAUA GAAUAGGGUUACAGAGUUG
DDX41	51428	CUAAGGGCAUUACGUAUGA GAACUAUGUACACCGGAUU UAAAGCCUGUGACCAUCAA CAACACCCAUUCAGAUCCA	"scrambled"	-	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA